

REMARKS

Claims 18, 19, 23-26, 28, 29, 31 and 39 are pending. Claims 19, 28, 29 and 31 have been withdrawn from consideration. Claims 18, 23-26 and 39 are under examination.

Response to Drawings Objections

The drawings have been amended to correct the informalities noted by Examiner. Specifically, Figure 2 has been amended to label the large panel in the lower right quadrant of the drawing sheet with the letter "D". The specification has been amended at page 9 to refer only to panels A through D of Fig. 2.

The specification has been amended at page 10 to delete the reference letters (A)-(F) that do not appear in Fig. 6.

The specification has also been amended at page 10 to refer to the two panels, A and B, of Figure 8.

Response to Specification Objections

(a) Description of the Drawings

The objection is overcome by the specification amendments described above.

(b) Hyperlinks

The specification has been objected to for including embedded hyperlinks or other forms of browser-executable code. The website citations at pages 16 and 17 were previously amended to render them non-executable. The citations have been further amended to substitute "<dot>" for "[dot]" to more closely conform to what is believed the appropriate procedure for disabling browser executable codes. Thus, the objection for browser executable code is believed overcome.

The specification has been further objected to on the basis that the website citations represent an improper incorporation by reference. To comprise an incorporation by reference, the words "incorporated by reference" or the like must be utilized. "Mere reference to another application, patent, or publication is not an incorporation of anything therein into the application containing such reference for the purpose of the disclosure required by 35 U. S.C. 112, first

paragraph.” MPEP608.01(p)1.A. (citing *In re de Seversky*, 474 F.2d 671, 177 USPQ 144 (CCPA 1973).

The subject matter of the website citations on pages 16 and 17 is not incorporated by reference. Thus, MPEP 608.01(p) does not apply, and the citations do not represent incorporations by reference of hyperlink or other browser executable code.

(c) Trademarks in the specification

The specification has been amended to identify trademarks.

(d) Correction of errors in the specification

The specification has been reviewed, and corrections have been made.

Response to Section 112 Rejections

The amendment of claim 18 overcomes the rejection as to the claim language “obtainable”.

The amendment to claims 23 and 25 overcomes the rejection as to the claim language “primary antibody”.

Claim 26 – Alleged Lack of Written Description and Enablement

A written description rejection and lack of enablement rejection has been made against “biological targeting devices”. However, the claim or claims implicated in the rejection are not specifically identified in the rejection. Claim 26 is directed to a “biological targeting device”. Thus, applicants will treat the rejections under Section 112 first paragraph as a rejection of claim 26.

There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed. *In re Wertheim*, 191 USPQ 90, 96 (CCPA 1976). MPEP 2163 II.A. A description as filed is presumed to be adequate, unless or until sufficient evidence or reasoning to the contrary has been presented by the examiner to rebut the presumption. See, e.g., *In re Marzocchi*, 169 USPQ 367, 370 (CCPA 1971). MPEP 2163.04. The examiner, therefore, must have a reasonable basis to challenge the adequacy of the written description. MPEP 2163.04.

Similarly, there is a strong presumption that the specification, which discloses how to make and use the invention, complies with the enablement requirement of the first paragraph of 35 U.S.C. 112, unless there is reason to doubt the objective truth of the specification. *In re Marzocchi, supra*. In establishing a prima facie basis to deny patentability under the first paragraph of 35 U.S.C. 112 for lack of adequate enabling support, it is incumbent upon the examiner to explain why he doubts the truth or accuracy of the supporting specification and to buttress assertions with evidence or cogent reasoning inconsistent with the specification. *In re Strahilevitz*, 212 USPQ 561 (CCPA 1975); *In re Bowen*, 181 USPQ 48 (CCPA 1947); *In re Marzocchi, supra*. "Any assertion that the enabling disclosure is not commensurate in scope with the protection sought must be supported by evidence or reasoning substantiating the doubts so expressed." *In re Dinh-Nguyen*, 181 USPQ 46, 47 (CCPA 1974).

As long as enough detail in the specification has been set forth to allow an ordinarily skilled in the art (1) to understand what is claimed and (2) to recognize that the named inventor(s) invented what is claimed, the written description of 35 U.S.C. § 112, first paragraph, has been satisfied. *Univ. of Rochester v. G.D. Searle & Co.*, 69 U.S.P.Q.2d 1886, 1896 (Fed. Cir. 2004).

The description needed to satisfy the written description requirements of 35 U.S.C. § 112 varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. MPEP 2163 II.A.3.(a)(i). In most technologies which are mature, and in which the knowledge and level of skill in the art is high, a written description question should not be raised for claims present in the application when originally filed. *Id.*

Moreover, what is conventional or well known to one of ordinary skill in the art need not be disclosed in detail to satisfy the written description requirement. *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 94 9Fed. Cir. 1986); *Capon v. Eshhar*, 76 USPQ2d 1078, 1085 (Fed. Cir. 2005) ("The 'written description' requirement must be applied in the context of the particular invention and the state of the knowledge... As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution"). MPEP 2163 II.A.3. "[A] patent applicant does not need to include in the specification that which

is already known to and available to one of ordinary skill in the art." *Koito Mfg. Co. v. Turn-Key-Tech LLC*, 381 F.3d 1142, 1156, 72 U.S.P.Q.2d 1190, 1200 (Fed. Cir. 2004).

The same reasoning applies to satisfaction of the enabling disclosure requirement of Section 112. "A patent need not disclose what is well-known in the art." *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). "A patent need not teach, and preferably omits, what is well-known in the art." *Spectra-Physics, Inc. v. Coherent, Inc.*, 3 U.S.P.Q.2d 1737, 1743 (Fed. Cir. 1987), cert. denied, 108 S.Ct. 346 (1987); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, *supra*. The specification may assume "that which is common and well-known" to persons skilled in the relevant art. *Webster Loom v. Higgins*, 105 U.S. 580 (1981). Also see M.P.E.P. 601. The invention as claimed in claim 26 satisfies both the written description and enabling disclosure requirements of Section 112.

Claim 26 clearly indicates the structural features of the biological targeting device namely: i) an antibody according to claim 18, or an antigen-binding fragment thereof, and, ii) a therapeutic ligand. The therapeutic ligand is taught at page 25 lines 27 to 29 to be suitable, for example, in radiotherapy. The skilled person would immediately understand claim 26 to be directed to a device wherein the specific antibody of claim 18 or an antigen binding fragment thereof can target a therapeutic ligand (a compound which can be used to provide a therapeutic effect), for example a therapeutic ligand suitable for radiotherapy. The antibody or fragment of claim 26 is clearly identifiable, and the deposit filed shows the Applicant was in the possession of the antibody at the time the application was filed.

Therapeutic ligands, for example, for use in radiotherapy would be known to the skilled artisan. Thus, the Applicant was in possession of the invention as claimed by claim 26 at the filing date. Attached is a review by McCarron *et al. Molecular Interventions* 5(6):368-380, Dec. 2005. The paper describes previously used therapeutic ligands in combination with antibody molecules. While the review was published after the filing date of the present application, details of particular examples known before the filing date are discussed and referenced within the paper (see reference 8 cited in column 2 of page 369, references 66 to 74 cited in table 1 of page 370 and references 45 and 46 cited on page 374 of the review article).

One of ordinary skill in the art would readily understand that effective delivery to the site of disease is a prerequisite for high efficacy and low toxicity of any drug substance. As illustrated by the documents cited in the attached review article, antibodies can facilitate the transport to and localization at a target site within the body of a conjugated drug cargo and thus invoke the "magic bullet" concept. As this increases the effective drug concentration at the target site, the therapeutic effect of the drug is optimized. Moreover, this may also allow the physician to lower the dose of the drug, which is of particular relevance if the drug has associated toxicities or if it is used in the treatment of chronic conditions.

Presently a number of approved antibody radiologic conjugates have been approved:

Zevalin (ibritumomab tiuxetan) - a monoclonal antibody conjugated with Tiuxetan (a radioactive isotope) that is approved for the treatment of B Cell Non-Hodgkin's lymphoma;

Bexxar (^{131}I - tositumomab) - a monoclonal antibody conjugated to a ^{131}I radioisotope that is approved for the treatment of lymphoma.

Thus, prior to the present invention, it was well-known to couple antibodies to therapeutic agents to target those agents to a body site expressing the antigen bound by the targeting antibody. What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail. *Hybritech Inc. v. Monoclonal Antibodies, Inc., supra*. The examiner does not have a reasonable basis to challenge either the enablement or the adequacy of the written description supporting claim 26, in view of the state of the art at the time of the invention. Reconsideration and withdraw of the written description rejection of claim 26 is respectfully requested.

Claims 23-25 Lack of Enabling Disclosure

A lack of enablement rejection has been made against claims directed to diagnostic kits containing antibody according to claim 18. However, the claim or claims implicated in the rejection are not specifically identified in the rejection. Claims 23-25 are directed to diagnostic kits containing the antibody according to claim 18. Thus, applicants will treat the rejection as a rejection of claims 23-25.

As indicated above, there is a strong presumption that the specification, which discloses how to make and use the invention, complies with the enablement requirement of the first paragraph of 35 U.S.C. 112, unless there is reason to doubt the objective truth of the specification. *In re Marzocchi, supra*. Any assertion that the enabling disclosure is not commensurate in scope with the protection sought must be supported by evidence or reasoning substantiating the doubts so expressed." *In re Dinh-Nguyen, supra*. The PTO has the burden of showing that the disclosure entails undue experimentation. *In re Angstadt*, 190 USPQ 214 (CCPA 1976). It is respectfully submitted that Examiner has not sustained this burden with respect to claims 23-25.

One of ordinary skill in the art would not have to perform undue experimentation to make and use the diagnostic kits of claims 23-25. The Examiner appears to be considering the application of the diagnostic kits to an assay to a bodily fluid, for example blood. But as indicated in the description, the assay is performed on tissue samples, for example immunohistochemical screening. As in such screening, the tissue tested would be known, it would not be necessary to differentiate between cells, as suggested by the Examiner. As evidenced by the teaching in the specification, using the antibody from ECACC Deposit No 03073001, of the tissues screened, only the tumor cells were found to express the antigen detected by the antibody from ECACC Deposit No 03073001 (MQ1). For the astrocytomas tested, 97% (29/30) were found to be MQ1 positive. No normal cells were found to be MQ1 positive.

The antibody from ECACC Deposit No 03073001 was able to distinguish between low grade, well differentiated pilocytic astrocytomas and non-neoplastic related gliosis. With the inherent underlying genetic instability of cancer, it seems unreasonable to preclude any diagnostic utility on the basis that only 97% of the tested tumor population expressed the antigen.

For primary breast carcinoma, 60% (137/228) tumors were found to be MQ1 positive. Again no normal cells or benign tissues were found to be MQ1 positive.

As evidenced by a subsequent paper (Fon *et al.*, *British Journal of Surgery* 93:309-314, 2006, coy enclosed) MQ1 expression was found to be significantly correlated with tumor grade, presence of lymphovascular invasion, the Nottingham Prognostic Index and the development of

local recurrence. This clearly indicates the utility of the present antibody and the methods as claimed.

As indicated above, as the antibody and the diagnostic methods as claimed may be utilized in assays, such as immunoassays, wherein the tissue type is known, even if normal tissues are determined to express MQ1, as suggested by the Examiner, using such immunohistochemical methods the tissues which are tested can be specifically selected.

It is respectfully submitted that Examiner has not sustained the burden of demonstrating that the subject matter of claims 23-25 fails to comply with the enabling disclosure requirement of Section 112. Practice of the claimed invention does not involve undue experimentation, given the state of the art at the time of the invention. Reconsideration and withdrawal of the lack of enablement rejection of claims 23-25 is respectfully requested.

Statement of Biological Deposit

A Supplemental Statement of Biological Deposit is submitted herewith containing the additional averments noted by Examiner

Response to Section 102 Rejection

The rejection is premised on a claim interpretation triggered by the claim language "antibody...obtainable" in claim 18. The amendment to claim 18 overcomes the rejection. The monoclonal antibody produced by the hybridoma ECACC 03073001 is not the same as any antibody of Shearman *et al.*

Response to Section 103 Rejection

Claims 23 and 25 are rejected as allegedly obvious over Shearman *et al.* in view of Monia *et al.* Shearman *et al.* does not disclose the antibody of claim 18, that is, the antibody produced by the hybridoma ECACC 03073001. Monia *et al.* does not remedy the deficiency in Shearman *et al.*, and does not render obvious the monoclonal antibody of claim 18. In view of the allowability of antibody claim 18, claims 23 and 25, which depend from claim 18, are likewise allowable.

Rejoinder

Applicants respectfully request rejoinder and allowance of all claims.

Conclusion

The claims remaining in the application are believed to be in condition for allowance. An early action toward that end is earnestly solicited.

Respectfully submitted,
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Fig. 1

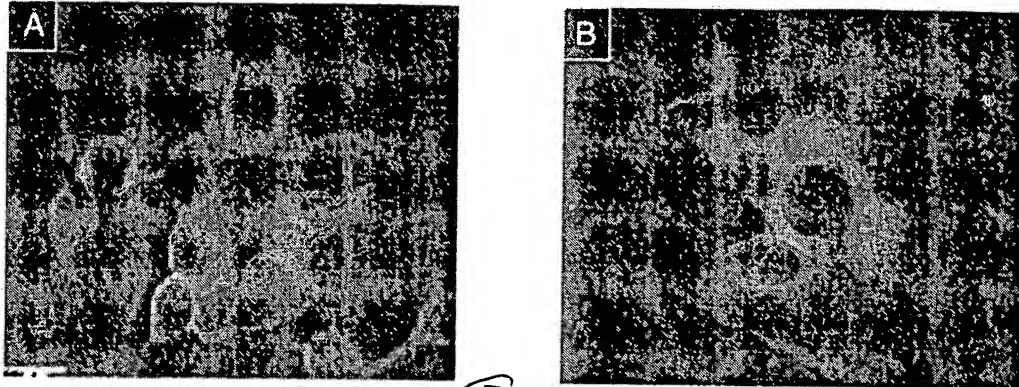
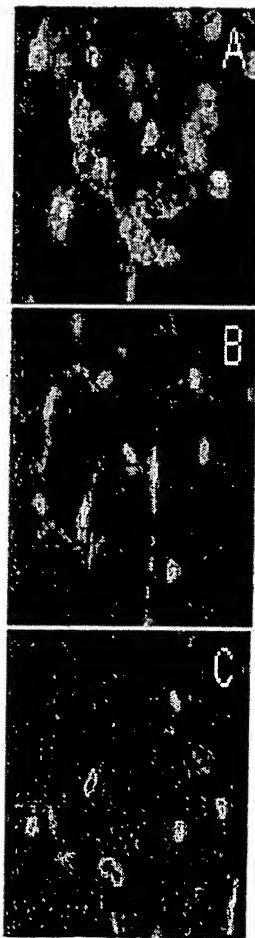
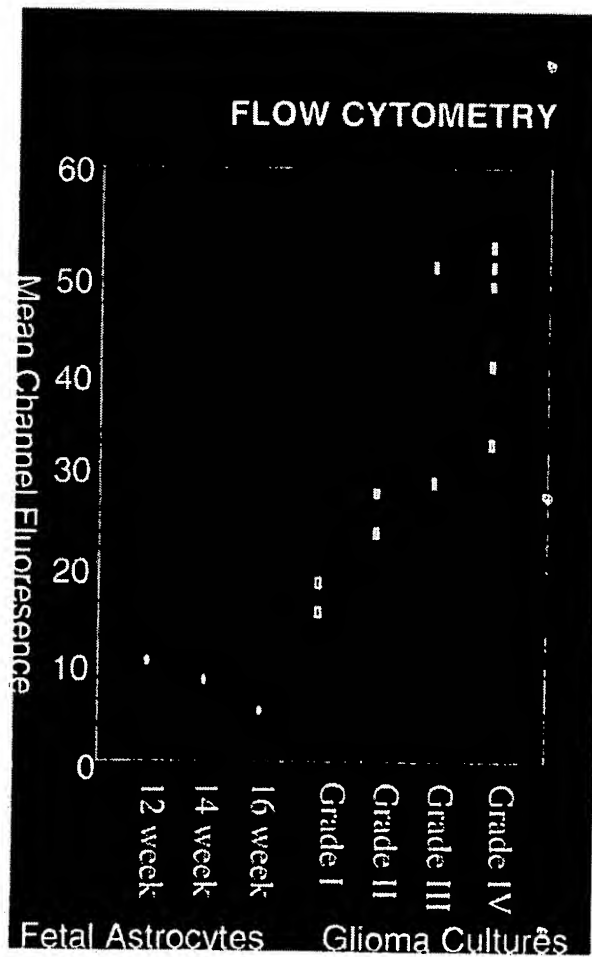


Fig. 2



D ← panel letter inserted



Prognostic significance of glycoprotein pMQ1 in breast cancer

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Background: A novel glycoprotein, pMQ1, is positively correlated with increasing histological grade in malignant astrocytomas. Cerebral metastases from breast cancer have also been found to contain pMQ1-positive cells. This study aimed to determine the role of pMQ1 in primary breast cancer.

Methods: Breast cancer specimens were analysed for pMQ1 by immunohistochemistry. The expression of pMQ1 was correlated with conventional prognostic indicators. Kaplan–Meier analyses were performed to compare clinical outcome between pMQ1-positive and pMQ1-negative tumours.

Results: pMQ1 was expressed in most of the breast cancer specimens. The surrounding normal tissue margins and benign breast tissues always lacked pMQ1 expression. A significant positive correlation was observed between pMQ1 expression and histological grade, the presence of lymphovascular invasion and Nottingham Prognostic Index. Cancers that were pMQ1 positive were significantly more likely to develop a local recurrence.

Conclusion: pMQ1 appears to be a tumour-associated protein. The positive correlation of pMQ1 with histological grade, presence of lymphovascular invasion and Nottingham Prognostic Index suggests that it confers an adverse prognosis.

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Introduction

In 1997, Mulligan and co-workers isolated a monoclonal antibody by inoculation of G-CCM cell line derived from an anaplastic astrocytoma¹. This antibody recognizes an epitope on an astrocytoma-associated glycoprotein, named pMQ1. N-terminal sequencing showed that pMQ1 was a previously undescribed mammalian protein that shared significant sequence homology with the gene product of a micro-organism (ORF6), implicated in cell adhesion². It was not expressed in normal tissue, diminished with increasing fetal astrocyte maturity and increased with higher histological grade in astrocytomas, suggesting it may be an oncofetal protein with adverse potential.

The expression of pMQ1 was not confined to astrocytomas but was also observed in metastatic cancer cells from a primary breast cancer. The major cause of treatment failure after surgical resection for breast cancer is the development of either a local or a distant recurrence^{3,4}. Biological characteristics of cancer cells may

help to predict the prognosis in women with breast cancer⁵. Current prognostic indicators such as age, tumour size, histological grade^{6,7}, axillary lymph node involvement⁸, presence of lymphovascular invasion⁹, oestrogen receptor (ER) status¹⁰, Ki-67^{11,12} and p53¹³ are being used to predict treatment outcome, but with limited accuracy^{14,15}. This project was initiated to assess the role of pMQ1 in primary breast cancer. The level of pMQ1 expression in breast cancer was correlated with known prognostic indicators.

Materials and methods

Patient and tumour characteristics

Archival specimens were retrieved from 228 women diagnosed with breast cancer between 1984 and 1998. This study received ethical approval from the Medical Ethics Committee of The Queen's University of Belfast. All women included in this study received surgical treatment by segmental resection or simple mastectomy,

and axillary dissection when indicated. Tumours were graded using the modified Scarf, Bloom and Richardson method^{16,17}.

Benign breast tissue

In this study, normal breast tissue was used as a control, as it provided a reference during the assessment and interpretation of pMQ1 expression in test samples. Normal breast tissue was obtained from patients who had undergone breast reduction or a diagnostic biopsy that turned out to be unremarkable histologically. A total of 25 control specimens along with five samples of benign fibrocystic disease of the breast and five fibroadenomas were retrieved from archival materials. Samples were treated identically to those from study subjects.

Primary antibodies and immunohistochemistry

Immunoreactivity for pMQ1 was detected using a monoclonal antibody to pMQ1, raised against surface proteins expressed by an anaplastic astrocytoma cell line, G-CCM¹. Immunohistochemical staining was performed on slides from routinely fixed paraffin-embedded blocks. The sections were pretreated in a microwave oven at 450 W for 3 min and incubated overnight at room temperature with pMQ1 primary antibody. The reaction was visualized using a streptavidin-biotin immunoperoxidase system (LSAB 2 kit; DAKO, Milan, Italy) and 3-amino-9-ethyl carbazole solution (DAKO) as chromogenic substrate. The tissue sections were counterstained with Mayer's haematoxylin and mounted in DPX resin. For negative controls, the primary antibody was replaced with mouse monoclonal IgM antibody with specificity for *Aspergillus niger* glucose oxidase (an enzyme not present or inducible in mammalian tissues).

Assessment of pMQ1 expression

Serial dilutions were performed to optimize the detection of pMQ1 antibody before its application on test specimens, and each time a new batch of monoclonal antibody was introduced. Two independent observers assessed pMQ1 expression on the tissue sections by light microscopy. The staining intensity and the proportion of pMQ1-positive tumours were assessed and scored as: 0, completely negative; 1+, weak; 2+, moderate; 3+, strong. Breast cancers with a pMQ1 staining intensity of 2 or 3 and proportion of pMQ1-positive tumour greater than 10 per cent were graded pMQ1 positive. A staining intensity score of 0-1, or less than 10 per cent proportion, was recorded as pMQ1 negative.

Analyses of oestrogen receptor, p53 and Ki-67

A monoclonal antibody to ER, mouse anti-ER (ER1D5; DAKO), a mouse antihuman anti-p53 monoclonal antibody (DAKO-p53) and polyclonal antihuman Ki-67 antibody (DAKO, Glostrup, Denmark) were used for the immunohistochemical analysis of ER status, p53 and Ki-67, respectively. The quickscore semiquantitative method was used in the assessment of ER status¹⁸. A score of 0 was recorded as ER negative, 1-6 as weakly ER positive and 7-18 as strongly ER positive. For p53 and Ki-67, a cut-off point was chosen at more than 6 to exclude weakly staining tumours. The cut-off for p53 positivity was set at more than 6 to exclude false-positive cases that may not carry the p53 mutation. Focal Ki-67-positive staining in less than 20 per cent of the tumour specimen was regarded as within the normal rate of tissue proliferation¹⁹.

Statistical analysis

Clinical and laboratory data were analysed using the Statistical Package for the Social SciencesTM version 9 (SPSS, Chicago, Illinois, USA)²⁰. The association between pMQ1 expression and the currently known prognostic indicators was evaluated using a bivariate Spearman's correlation analysis and χ^2 test. The kappa statistical method was performed to measure the level of agreement between two observers²¹ to ensure that the degree of inter-observer variation fell within acceptable limits.

The results of pMQ1 analysis were expressed as either pMQ1 positive or pMQ1 negative. The pMQ1 status was then correlated with the patients' clinical outcome, as defined by the development of either a local or a distant recurrence, disease-free survival, event-free survival and overall survival. The difference in clinical outcome between pMQ1-positive and pMQ1-negative cancers was analysed using the Kaplan-Meier method using the cumulative hazard option, and the separation in survival curves tested for statistical significance using log rank analysis.

Results

In normal breast tissue, the staining intensity for pMQ1 was either absent or weak, distributed basally in contact with the basement membrane. A similar staining pattern was observed in fibroadenomas and benign fibrocystic disease of the breast. All benign breast tissue lacked pMQ1 expression. Similarly, normal or uninvolved tissue at the margin of breast cancer was also negative for pMQ1 expression and served as a negative internal control.

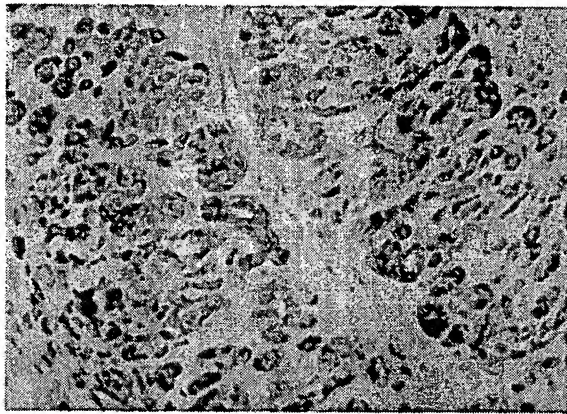


Fig. 1 pMQ1 immunohistochemistry in breast cancer tissue showing cell surface membrane and intracytoplasmic staining throughout the glandular component of invasive ductal carcinoma (original magnification $\times 60$)

Sixty per cent (137/228) of the archival breast cancer specimens were pMQ1 positive. The staining pattern for pMQ1 was distributed at the cell surface membrane and intracytoplasmic regions; pMQ1 staining was either focal or diffuse. In most cases, pMQ1 was diffusely expressed throughout the entire cancer ($n = 94$) (Fig. 1). Ductal carcinoma *in situ*, in which the cancer cell proliferation was confined to the duct without evidence of a stromal invasion, was also found to be positive for pMQ1. Lymph

nodes that contained breast cancer metastases expressed pMQ1 in a pattern similar to the primary cancer. In all pMQ1-positive lymph nodes, the distribution of pMQ1 immunostaining was confined to the cancer cells.

Correlation between pMQ1 and prognostic indicators

Histological grade

Among 225 tumours where histological grading was possible, 44 (19 per cent) were grade I, 103 (45 per cent) grade II and 78 (34 per cent) grade III. Most of the histological grade III breast cancers were pMQ1 positive (74.4 per cent, 58/78). There was a positive correlation between pMQ1 expression and increasing histological grade (Spearman's correlation coefficient $r = 0.271$, $P < 0.001$, Table 1). Most of the tumours were invasive ductal carcinoma (72 per cent, $n = 166$). A bivariate correlation analysis revealed a significant positive association between pMQ1 expression and increasing histological grade in this subgroup of patients (Spearman's correlation coefficient $r = 0.264$, $P = 0.001$).

Lymphovascular invasion

Lymphovascular invasion was observed unequivocally in 66 of 169 (39 per cent) tumours studied and was positively correlated with higher histological grade tumours ($r = 0.018$, $P < 0.001$). pMQ1 was also expressed in a significantly higher number of tumours with

Table 1 Correlation between pMQ1 expression and primary prognostic indicators in human breast cancer

Variable	Category	<i>n</i>	pMQ1-positive tumours (%)	Correlation coefficient, <i>r</i>	<i>P</i>
Age (years) ($n = 228$)	< 40	56	70.0	-0.135	0.042
	41-55	55	62.0		
	56-70	88	55.0		
	> 70	31	55.0		
Tumour size ($n = 227$)	T1: < 2 cm	73	60.3	0.227	0.027
	T2: 2-5 cm	139	69.8		
	T3: > 5 cm	15	69.2		
Histological grade ($n = 225$)	Low (grade I)	44	36.4	0.271	< 0.001
	Moderate (grade II)	103	58.3		
	High (grade III)	78	74.4		
Histological subtype ($n = 228$)	Invasive ductal carcinoma (not otherwise specified)	166	64.0	0.182	0.018
	Invasive lobular carcinoma	42	43.0		
	Other	20	55.0		
Lymphovascular invasion ($n = 169$)	Present	66	74.2	0.523	0.523
	Absent	103	56.3		
Lymph node status ($n = 174$)	No nodes involved	93	66.0	0.523	0.523
	1-3 nodes involved	51	67.0		
	≥ 4 nodes involved	30	67.0		

*Bivariate Spearman's correlation analyses.

Table 2 Correlation between pMQ1 and secondary prognostic variables in human breast cancer

Variable	Category	n	pMQ1 expression (%)	Correlation coefficient, <i>r</i>	<i>P</i>
Oestrogen receptor (<i>n</i> = 142)	ER negative	58	79	-0.278	0.001
	ER weakly positive	21	71		
	ER strongly positive	63	51		
Ki-67 (<i>n</i> = 129)	Positive	68	63	0.387	0.387
	Negative	61	70		
p53 (<i>n</i> = 125)	Positive	75	75	0.878	0.878
	Negative	50	74		

ER, oestrogen receptor. *Bivariate Spearman's correlation analyses.

lymphovascular invasion (74 per cent, 49/66, $r = 0.182$, $P = 0.018$, Table 1).

Age

An inverse correlation existed between pMQ1 and age (Spearman's correlation coefficient $r = -0.135$, $P = 0.042$). Cancers that were pMQ1 negative were more common in older women.

Oestrogen receptor status

ER status was evaluated in 142 breast cancer specimens. Eighty-four (59 per cent) tumours were ER positive and the remainder were ER negative. There was a significant inverse correlation between pMQ1 expression and ER status (Spearman's correlation coefficient $r = -0.278$, $P = 0.001$). The ER-positive breast cancers were less likely to express pMQ1 (Table 2).

Nottingham Prognostic Index

The Nottingham Prognostic Index (NPI) combines several variables known to have a significant impact on the clinical outcome²²⁻²⁴. Results for pMQ1 expression and NPI were recorded for 171 women in this study. The NPI was significantly correlated with pMQ1 expression (Spearman's correlation coefficient $r = 0.201$, $P = 0.008$). Cancers that were pMQ1 positive were more common among patients who had a higher NPI.

Tumour size, lymph node status, Ki-67, p53

No significant correlation was observed between pMQ1 and tumour size, lymph node status, Ki-67 and p53, or tumour node metastasis (TNM) stage²⁵.

Follow-up

The patients were followed for evidence of local or distant recurrences. Follow-up was complete in 211 patients; 17 were either lost to follow-up or data were inaccurate. A total of 15 per cent (28/211) of the women developed a

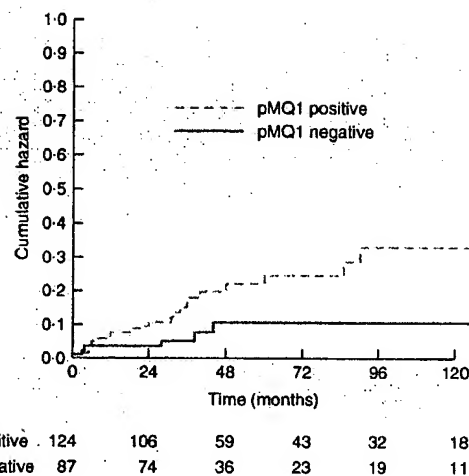


Fig. 2 Kaplan-Meier cumulative hazard analysis comparing the risk of local recurrence by pMQ1 expression ($P = 0.036$; log rank test). The risk of developing a local recurrence was significantly higher in women with pMQ1-positive cancers

local recurrence at the site of the primary tumour. A total of 23.9 per cent (53/211) died from distant recurrence. Kaplan-Meier analysis using the cumulative hazard option revealed that local recurrence was significantly more common in pMQ1-positive tumours (22/124) than pMQ1-negative tumours (6/87, $P = 0.036$, Fig. 2), but that the rate of distant tumour recurrence was similar in the two groups ($P = 0.677$). Kaplan-Meier survival plots generated for disease-free survival, event-free survival and overall survival between pMQ1-positive and pMQ1-negative patients did not show any statistically significant differences ($P = 0.130$, $P = 0.170$ and $P = 0.847$, respectively).

Discussion

Cancer cells possess the ability to invade mesenchyme by producing proteins that stimulate angiogenesis into

the tumour mass²⁶. Mulligan *et al.* isolated a monoclonal antibody that recognized an epitope on an astrocytoma glycoprotein named pMQ1²⁷, which shared significant sequence homology with a gene product of a micro-organism implicated in cell adhesion². This suggests that pMQ1 could be a cell adhesion molecule or receptor.

This study found that pMQ1 was expressed in 60 per cent of primary human breast cancer specimens, ductal carcinoma *in situ* and axillary lymph node metastases. Normal breast tissue and benign breast diseases (fibroadenoma, fibrocystic disease) lacked pMQ1 expression.

The presence of pMQ1 in breast and astrocytoma cancer cells suggests that it is a tumour-associated protein. Other tumour-associated markers have found clinical application in the diagnosis, detection and monitoring of cancer progression^{28–31}. In a practical setting, pMQ1 may have potential as an additional tool for the histological diagnosis and detection of minimal residual disease in women with breast cancer.

The expression of pMQ1 had a positive correlation with increasing histological grade in this study. pMQ1 staining was more common among histological grade III (poorly differentiated) cancers. This observation concurs with earlier findings in astrocytomas^{1,27} and suggests pMQ1 may regulate cellular dedifferentiation.

Lymph node status is a known predictor of distant metastases in breast cancer⁸. In this study, pMQ1 expression was not significantly associated with lymph node status but was strongly correlated with the presence of lymphovascular invasion, which is required for the development of axillary lymph node metastases. A possible explanation is that pMQ1 may be involved in the early events of lymphatic spread before the overt development of axillary lymph node metastases³².

There was also a significant inverse correlation between pMQ1 expression and ER status ($P = 0.001$). The aggressive tumours were more likely to be pMQ1 positive and ER-negative. A critical milestone in the development of breast cancer is the emergence of oestrogen hormone independence, associated with its metastatic potential³³. The ER is always present in normal breast tissue and is usually positive in well differentiated breast cancers. Aggressive breast cancers are more likely to be ER negative³⁴. *In vitro* and mouse model studies have shown that the lack of ER on cancer cells is associated with increased basement membrane invasiveness. The acquisition of oestrogen-independent phenotype could be associated with an increased ability of cancer cells to proliferate and migrate³³.

The NPI^{23,24} and TNM staging system²⁵ are both derived by combining several prognostically significant variables. The principal difference between them is that the TNM staging system does not consider histological grade. pMQ1 staining was significantly correlated with increasing histological grade. In this study, pMQ1 expression was found to have a significant positive correlation with the NPI but not with TNM stage. Therefore, the apparent strong association between pMQ1 and NPI may in fact be a reflection of the strong correlation between pMQ1 expression and the histological grade.

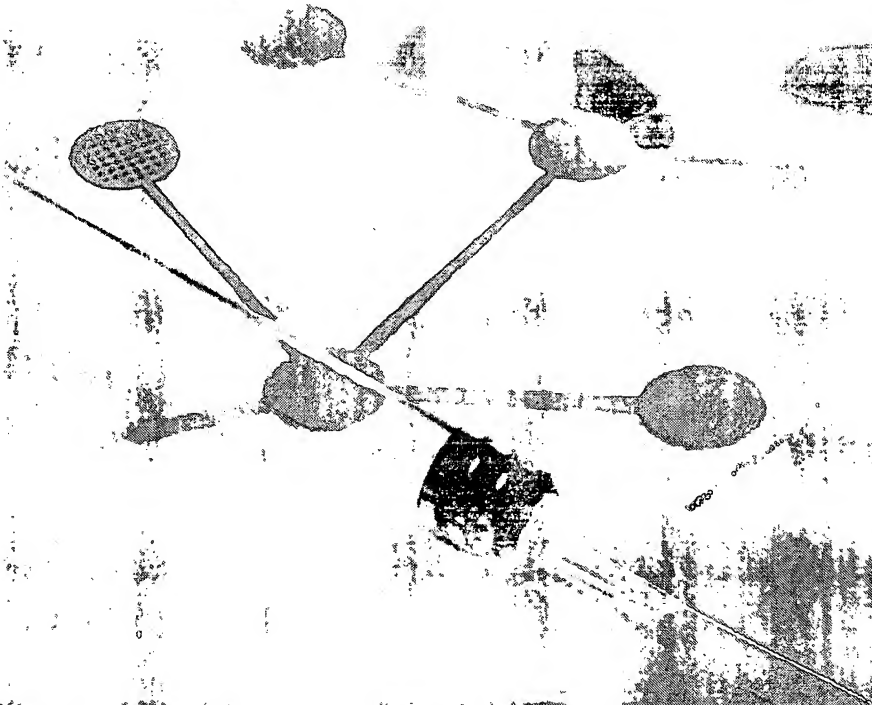
Women with pMQ1-positive cancers had a significantly higher cumulative risk of local recurrence. Local recurrence typically develops within the first 2–3 years after surgery and leads to an increased risk of distant metastases³⁵. It is possible that pMQ1 could be used to select women at high risk of developing a recurrence for more radical treatment.

References

- Mulligan K, McCormick D, Dermott E, Wisdom GB. A 'new' glycoprotein expressed on the surface of human glioma cells *in vitro*. *Br J Neurosurg* 1997; 11: 171 (Abstract).
- Layh-Schmitt G, Herrmann R. Localization and biochemical characterization of the ORF6 gene product of *Mycoplasma pneumoniae* P1 operon. *Infect Immun* 1992; 60: 2906–2913.
- Berg JW, Hutter RV. Breast cancer. *Cancer* 1995; 75(Suppl): 257–269.
- Bremers AJA, Rutgers EJ, van de Velde CJH. Cancer surgery: the last 25 years. *Cancer Treat Rev* 1999; 25: 333–353.
- Volpi A, De Paola F, Nanni O, Granato AM, Bajorko P, Becciolini A *et al.* Prognostic significance of biological markers in node negative breast cancer patients: a prospective study. *Breast Cancer Res Treat* 2000; 63: 181–192.
- Henson DE, Ries L, Freedman LS, Carriaga M. Relationship among outcome, stage of disease, and histological grade for 22 616 cases of breast cancer. The basis for a prognostic index. *Cancer* 1991; 68: 2142–2149.
- Genestie C, Zafrani B, Asselain B, Fourquet A, Rozan S, Validire P *et al.* Comparison of the prognostic value of Scarff-Bloom-Richardson and Nottingham histological grades in a series of 825 cases of breast cancer: major importance of the mitotic count as a component of both grading systems. *Anticancer Res* 1998; 18: 571–576.
- Weigand RA, Isenberg WM, Russo J, Brennan MJ, Rich MA. Blood vessel invasion and axillary lymph node involvement as prognostic indicators for human breast cancer. *Cancer* 1982; 50: 962–969.
- Pinder SE, Ellis IO, Galea M, O'Rourke S, Blamey RW, Elston CW. Pathological prognostic factors in breast cancer. III. Vascular invasion: relationship with recurrence and

- survival in a large study with long-term follow-up. *Histopathology* 1994; 24: 41–47.
- 10 Winstanley J, Cook T, George WD, Murray G, Holt S, Croton R *et al.* The long term prognostic significance of oestrogen receptor analysis in early carcinoma of the breast. *Br J Cancer* 1991; 64: 99–101.
 - 11 Vielh P, Chevillard S, Mosseri V, Donatini B, Magdelenat H. Ki67 index and S-phase fraction in human breast carcinomas. Comparison and correlations with prognostic factors. *Am J Clin Pathol* 1990; 94: 681–686.
 - 12 Haerslev T, Jacobsen GK, Zedeler K. Correlation of growth fraction by Ki-67 and proliferating cell nuclear antigen (PCNA) immunohistochemistry with histopathological parameters and prognosis in primary breast carcinomas. *Breast Cancer Res Treat* 1996; 37: 101–113.
 - 13 Kato T, Kameoka S, Kimura T, Tanaka S, Nishikawa T, Kobayashi M. p53, mitosis, apoptosis and necrosis as prognostic indicators of long-term survival in breast cancer. *Anticancer Res* 2002; 22: 1105–1112.
 - 14 Garne JP, Aspegren K, Linell F, Rank F, Ranstam J. Primary prognostic factors in invasive breast carcinoma with special reference to ductal carcinoma and histologic malignancy grade. *Cancer* 1994; 73: 1438–1448.
 - 15 Thomssen C, Jänicke F, Kaufmann M, Scharl A, Hayes DF. Do we need better prognostic factors in node-negative breast cancer? *Eur J Cancer* 2000; 36: 293–306.
 - 16 Bloom HJ, Richardson WW. Histological grading and prognosis in breast cancer: a study of 1049 cases, of which 359 have been followed for 15 years. *Br J Cancer* 1957; 11: 359–377.
 - 17 Frierson HF Jr, Wolber RA, Berean KW, Franquemont DW, Gaffey MJ, Boyd JC *et al.* Interobserver reproducibility of the Nottingham modification of the Bloom and Richardson histologic grading scheme for infiltrating ductal carcinoma. *Am J Clin Pathol* 1995; 103: 195–198.
 - 18 Detre S, Saccani Jotti G, Dowsett M. A 'quickscore' method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. *J Clin Pathol* 1995; 48: 876–878.
 - 19 van Diest PJ, van Dam P, Henzen-Longmans SC, Berns E, van der Burg ME, Green J *et al.* A scoring system for immunohistochemical staining: consensus report of the task force for basic research of the EORTC–GCCG. European Organization for Research and Treatment of Cancer–Gynaecological Cancer Cooperative Group. *J Clin Pathol* 1997; 50: 801–804.
 - 20 Nourousi MJ. *SPSS Statistical Software* SPSS, Chicago, 1994.
 - 21 Altman DG. *Practical Statistics for Medical Research*. Chapman & Hall: London, 1991.
 - 22 Galea MH, Blamey RW, Elston CE, Ellis IO. The Nottingham Prognostic Index in primary breast cancer. *Breast Cancer Res Treat* 1992; 22: 207–219.
 - 23 Balslev I, Axelsson CK, Zedeler K, Rasmussen BB, Carstensen B, Mouridsen HT. The Nottingham Prognostic Index applied to 9149 patients from the studies of the Danish Breast Cancer Cooperative Group (DBCG). *Breast Cancer Res Treat* 1994; 32: 281–290.
 - 24 D'Eredita G, Giardina C, Martellotta M, Natale T, Ferrarese F. Prognostic factors in breast cancer: the predictive value of the Nottingham Prognostic Index in patients with a long-term follow-up that were treated in a single institution. *Eur J Cancer* 2001; 37: 591–596.
 - 25 Hermanek P, Hutter RVP, Sobin LH, Spiessl B, Wagner G. *TNM Atlas: Illustrated Guide to the TNM/pTNM Classification of Malignant Tumours*. UICC International Union Against Cancer (4th edn). Springer: Berlin, 1997.
 - 26 Hendrix MJ, Seftor EA, Kirschmann DA, Seftor RE. Molecular biology of breast cancer metastasis. Molecular expression of vascular markers by aggressive breast cancer cells. *Breast Cancer Res* 2000; 2: 417–422.
 - 27 Mulligan KA, Gillespie H, Mirakhor M, McCormick D. Characterization of a novel oncofetal cell surface glycoprotein. *Neuropathol Appl Neurobiol* 2002; 28: 157 (Abstract).
 - 28 Kiang DT, Greenberg LJ, Kennedy BJ. Tumor marker kinetics in the monitoring of breast cancer. *Cancer* 1990; 65: 193–199.
 - 29 Hayes DF. Tumor markers for breast cancer. Current utilities and future prospects. *Hemat Oncol Clin N Am* 1994; 8: 485–506.
 - 30 Ballesta AM, Molina R, Filella X, Jo J, Gimenez N. Carcinoembryonic antigen and staging and follow-up of patients with solid tumors. *Tumour Biol* 1995; 16: 32–41.
 - 31 McCoy JL, Rucker R, Petros JA. Cell-mediated immunity to tumor-associated antigens is a better prognostic predictor of survival in early stage breast cancer than stage, grade or lymph node status. *Breast Cancer Res Treat* 2000; 60: 227–234.
 - 32 Kinoshita J, Kitamura K, Kabashima A, Saeki H, Tanaka S, Sugimachi K. Clinical significance of vascular endothelial growth factor-C (VEGF-C) in breast cancer. *Breast Cancer Res Treat* 2001; 66: 159–164.
 - 33 Thompson EW, Paik S-Y, Brunner N, Sommers CL, Zugmaier G, Clarke R *et al.* Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cell lines. *J Cell Physiol* 1992; 150: 534–544.
 - 34 Elledge RM, Osborne CK. Oestrogen receptors and breast cancer. *BMJ* 1997; 314: 1843–1844.
 - 35 Veronesi U, Marubini E, Del Vecchio M, Manzari A, Androlea S, Greco M *et al.* Local recurrences and distant metastases after conservative breast cancer treatments: partly independent events. *J Natl Cancer Inst* 1995; 87: 19–27.

Antibody Conjugates and Therapeutic Strategies



Immunotherapeutics represent the largest group of molecules currently in development as new drug entities. These versatile molecules are being investigated for the treatment of a range of pathological conditions including cancer, infectious and inflammatory diseases. Antibodies can be used to exert biological effects themselves or as delivery agents of conjugated drug molecules. Site-specific delivery of therapeutic agents has been an ultimate goal of the pharmaceutical industry in order to maximize drug action and minimize side effects. Antibodies have the potential to realize this objective and in this review we will examine some of the main strategies currently being employed for the development of these diverse therapeutic molecules.

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INTRODUCTION

Antibodies represent the single largest class of new drug entities under development at this time. There are at least twenty immunotherapeutics currently marketed, with some 150 developmental products currently in clinical evaluation (1). The possibility of using antibodies as therapeutics was considered almost as early as immunologists came to understand their role as our body's natural defense against foreign agents. This potential, however, was only initially realized with the development of monoclonal antibodies (MAbs) in the 1970s (2). Immortalization of murine antibody-secreting B cells by hybridoma technology and their continual cell culture permitted large scale production of a single, pure antibody species, a prerequisite for therapeutic development. Initial attempts to develop antibody-based therapeutics encountered difficulties when it became clear that the human body recognized the murine MAbs as foreign molecules and developed an antibody-mediated immune response to clear these from the body (3). The development of these human anti-murine antibodies (HAMA) seriously reduced the half-life of the therapeutic MAbs in the blood stream, particularly with prolonged administration, rendering them useless as drug strategies.

In order to reduce the HAMA response to administered MAbs, protein engineers manipulated the nucleotide sequences of the antibodies to convert murine-like antibodies to more human-like antibodies. Techniques now used routinely for the "demurization" of MAb include complementarity-determining region (CDR, the region of an antibody that is most involved in binding an antigen) grafting, humanization, and even the production of transgenic mice that make fully human antibodies. These advances in antibody engineering have created a surge in development of antibody-based therapeutic agents such that within the global pharmaceutical industry, larger companies, once slow to embrace antibody technology, have moved to secure intellectual property (IP). The securing of IP has been achieved by large licensing deals, takeovers, and mergers with biotechnology companies who offer novel antibody technology platforms or promising lead antibody drug candidates (4).

Antibody therapeutics exert their biological effects by a number of different mechanisms. Two possible mechanisms operate through the involvement of the immune system to induce cytotoxicity of the target cell population. The first of these is antibody-dependent cellular cytotoxicity (ADCC), where immune effector cells are recruited to the disease by virtue of the antibody Fc chain site once the antibody has bound to its target (5); however, the relevance of this mechanism, at least in clinical oncology, remains controversial (6). Alternatively, the Fc portion of a therapeutic antibody, as in the case of the CD20-specific rituximab (7), can recruit the activation of the complement cascade, resulting in the formation of the membrane attack complex, a mechanism referred to as complement-dependent cytotoxicity (CDC). Antibodies can also be used to modulate signaling pathways within the targeted cells. Bevacizumab (Avastin®) blocks angiogenesis by binding and sequestering soluble vascular endothelial growth factor (VEGF), and trastuzumab (Herceptin®) binds to

the cell surface protein HER2/neu, inhibiting proliferation pathways that are transduced through this signaling receptor.

In addition to these mechanisms of utilizing the antibody as the effector molecule, increasing attention has turned to the possibilities of using antibodies as delivery conduits and as a means to targeting disease sites specifically. By selectively targeting the drug to the site of disease, an antibody can induce the desired biological effects with improved therapeutic index. To date, three antibody conjugate therapies have been approved for marketing, namely, the radioimmunoconjugates ibritumomab tiuxetan (Zevalin®) and [¹³¹I]-tositumomab (Bexxar®) for the treatment of lymphoma and the drug conjugate gemtuzumab ozogamicin (Mylotarg®) for the treatment of acute myeloid leukemia. In this review, the different strategies employed in the production and application of antibody conjugates are discussed.

ANTIBODY CONJUGATES: THEORY AND APPLICATION

Effective delivery to the site of disease is a prerequisite for high efficacy and low toxicity of any drug substance. It is clear that antibodies can participate in this context by facilitating the transport of a drug cargo within the body and thereby invoking the often cited "magic bullet" concept, as put forward by Ehrlich over a century ago. Conjugation of a drug to an antibody makes it possible to achieve excellent localization of the drug at the desired site within the body (8). This increases the effective drug concentration within this target area, thereby optimizing the therapeutic effect of the agent. Furthermore, with targeted delivery, the clinician may be able to lower the dose of the therapeutic agent—something that is particularly relevant if the drug payload has associated toxicities or if it is to be used in the treatment of chronic conditions.

There are several basic considerations to be addressed when designing and applying an antibody conjugate to a particular disease model. The choice of drug payload is critical to ensure desired efficacy towards the targeted disease and that the stoichiometry, orientation, or associated chemistry of conjugation to the antibody will not hinder its biological activity. Furthermore, the design of any immunotherapeutic agent centers on the selection of antigens or biomarkers that are specific and accessible to antibody binding at the disease site. Not surprisingly, biomarker identification and characterization is a key focus for pharmaceutical and biotechnology companies alike and relies heavily on technologies such as DNA microarrays and proteomic techniques. Consequently, companies rapidly secure IP around newly identified biomarkers that could possibly lead to antibody-based drugs.

Despite substantial investment over the last ten years in biomarker identification, the number of therapeutically exploitable disease biomarkers that have been discovered is somewhat disappointing. This has led to renewed thinking as to how to examine novel strategies that can maximally exploit this small group of biomarkers, particularly when the biomarker is not a drug target. Antibody-drug

conjugates are an ideal way to utilize these identified biomarkers that cannot be targeted therapeutically by naked antibodies alone. We discuss below the main conjugate classes under investigation, together with those that have the potential to emerge as effective therapeutic agents against cancer and a wide range of other disease conditions.

PEPTIDE AND PROTEIN CONJUGATES

Numerous peptides and proteins elicit desirable therapeutic effects, but frequently utilization in therapeutic approaches have met with limited success owing to possible toxicity in the systemic circulation, poor bioavailability, degradation, and an inability to achieve useful therapeutic measures. Conjugation of these proteins to a suitable antibody or antibody fragment can circumvent these problems, achieving greater stability and higher specificity in delivery to the site of disease (9).

Peptides and proteins can be attached to each other by either chemical or recombinant means. The earliest examples of enzyme-antibody conjugates used chemical cross-linkers, such as maleimide, to couple thiol moieties between cysteine residues on each partner (10). This nonspecific approach can result in multiple products, with one or both partners inactivated by the coupling reaction. Alternative methods involve the specific conjugation of particular residues in the protein species, such as the prior oxidation of N-terminal threonine residues to produce a reactive aldehyde group that could then be specifically modified using cross-linking reagents (11). Currently, however, the most popular methodology uses recombinant DNA techniques to fuse the antibody open-reading frame to that of the therapeutic protein, giving rise to an antibody-protein fusion after translation. This approach to conjugation is much more amenable to the manufacture of a uniform protein species for therapeutic application (12). Frequently, the optimal fusion of protein entities can be a case of trial and error, often necessitating the incorporation of a short flexible linker to ensure maintenance of the two distinct biological activities. When full immunoglobulin G (IgG) antibodies are used, the most common strategy is the attachment of the cytokine at the C terminus of the antibody heavy chain (13), whereby with single chain antibody fragments (scFv), successful fusions have been achieved at both the N and C termini.

A particular antibody-therapeutic protein strategy that has been investigated by several groups is the selective activation of anticancer prodrugs by enzymes conjugated to a delivery antibody, frequently referred to as antibody-directed enzyme prodrug therapy (ADEPT) (14). This strategy has been investi-

gated in the treatment of solid tumors, whereby an antibody-enzyme conjugate is administered systemically, where it clears from the circulation and localizes to its target by virtue of the antibody binding to its specific biomarker on the tumor. Subsequently, a suitable non-toxic prodrug is then administered, which can be converted to its active cytotoxic form by the enzyme attached to the antibody. The activated drug penetrates the tumor cells and exerts its lethal effect in a localized manner (Figure 1). The ADEPT strategy facilitates the specific targeting of the disease site and directs toxicity to the target cell population. Crucially, this approach has the ability to target adjacent diseased cells not directly labelled by the antibody-enzyme conjugate and permits penetration of the free drug into the tumor mass. This effect is lacking during naked antibody or antibody-toxin conjugate solid tumor therapeutics, which can be viewed as a potential drawback to these other approaches.

In the design of an ADEPT approach, several factors must be considered. First, the choice of a suitably specific antibody and its cognate antigen are paramount. The chosen antigen is normally on the tumor surface itself, but can also be on surrounding tissue in some instances. An exquisite example of this is the targeting of the extra domain-B (ED-B) of fibronectin by L19, a recombinant antibody fragment (15). ED-B is specifically expressed in large amounts on neovasculature as a result of strongly pro-apoptotic stimuli secreted by many aggressive solid tumors. L19 localizes at these sites in a number of tumor models and has been used to deliver a wide range of therapeutic conjugates. The second consideration is the choice of enzyme to be attached to the antibody species. To add specificity to the activation of the prodrug, it would be pertinent to select a catalytic activity that would not be found at the target site—for example, an activity originating from a foreign organism, such as bacterial β -lactamase or carboxypeptidase G2. Alternatively, mammalian enzymes that are unlikely to be found in high basal concentrations in the body, such as carboxypeptidase A and β -glucuronidase, have also been used. Both strategies can ensure effective activation of the prodrug but are not without their drawbacks. Although high specificity can be obtained with bacterial or fungal enzymes, severe immunogenicity towards these foreign proteins is

Table 1. Selected Enzymes and Pro-drug Combinations Used in ADEPT Strategies

Enzyme	Reactivity	Pro-drug	Reference
Alkaline phosphatase	Hydrolysis of phosphate groups	Etoposide	(66)
		Doxorubicin	(67)
		Phenol mustard	(68)
Carboxypeptidase G2	Cleavage between glutamyl moiety and aromatic nucleus	Nitrogen mustards	(14)
β -lactamase	Cleavage of lactam ring: elimination of substituents attached to 3' of cephalosporin derivatives	Doxorubicin	(69)
		Paclitaxel	(70)
		Mitomycin	(71)
Cytosine deaminase	Deamination of cytosine to uracil	5-fluorouracil	(72)
Carboxypeptidase A	Cleavage of α -glutamyl peptides	Methotrexate	(73)
		Antifolates	(74)

an important concern. Problems of immunogenicity may be avoided by using mammalian enzymes; however, because similar (mammalian) catalytic activities are likely to be present in the patient, the specificity of prodrug activation could be confounded by activation at sites other than the intended target region. The final component of an ADEPT approach is the actual prodrug itself, which must be compatible substrate of the activating enzyme. Catalytic activation of prodrugs has long been a major focus of pharmaceutical research (16). Methotrexate prodrugs activated by carboxypeptidase A, etoposide phosphates by alkaline phosphatase and acid mustard compounds by carboxypeptidase G2 are just a few of the more common examples. Given the plethora of enzymes that occur in nature and the numerous choices of potential prodrug formats, a broad range of ADEPT strategies have been investigated (Table 1).

Enzymes are not the only protein-based conjugates that have been investigated as immunoconjugates. Cytokines and other small proteins that can elicit a strong biological response are attractive as therapeutic agents. Attachment of such molecules to antibodies can

improve their stability and plasma half-lives and facilitate accurate delivery. These improved parameters are particularly important given the exceptionally strong biological effects these proteins can elicit and the risk of possible unwanted side-effects following systemic delivery. Antibody-cytokine conjugates, or immunocytokines, have attracted particular interest for tumor treatment because of their potential to cause localized stimulation and activation of immune effector cells. Tumor necrosis factor- α (TNF- α), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon γ (IFN γ) and interleukin-2 (IL-2) are just a few of the cytokines that have been investigated in immunocytokine approaches (17). Reisfeld and coworkers have focused on IL-2, a pleiotropic cytokine that stimulates the activation of T-cells, natural killer (NK) cells, and macrophages, to enhance these cells recognition and destruction of tumor cells. From as early as the 1980s, the potency of IL-2 was appreciated, but with systemic delivery, an exploitable therapeutic index was not achievable. To localize delivery of the cytokine, this group fused IL-2 to an antibody directed towards disialoganglioside GD2, a gan-

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glioside that is over-produced in melanomas. In animal models, this immunocytokine afforded protection against the development of lung and hepatic metastases from injected melanomas (18). Further study confirmed that the protection mechanism was reliant on activation and recruitment of T cells to the tumor site. It has been postulated that IL-2 provides a co-stimulatory signal for the activation of CD8+ T cells which, in combination with signals elicited by antigenic peptides displayed on the MHC-I molecules of the tumor cells, are able to proliferate and eradicate the tumor. The humanized version of this antibody-IL-2 conjugate is currently in clinical trials for treatment of melanoma (19).

TNF- α is toxic to tumor cells, exerting its activity on endothelial cells of the tumor neovasculature. As is the case with IL-2, systemic delivery of TNF- α is not possible owing to its high toxicity to normal tissue. Neri and coworkers have conjugated TNF- α to their L19 fibronectin-specific

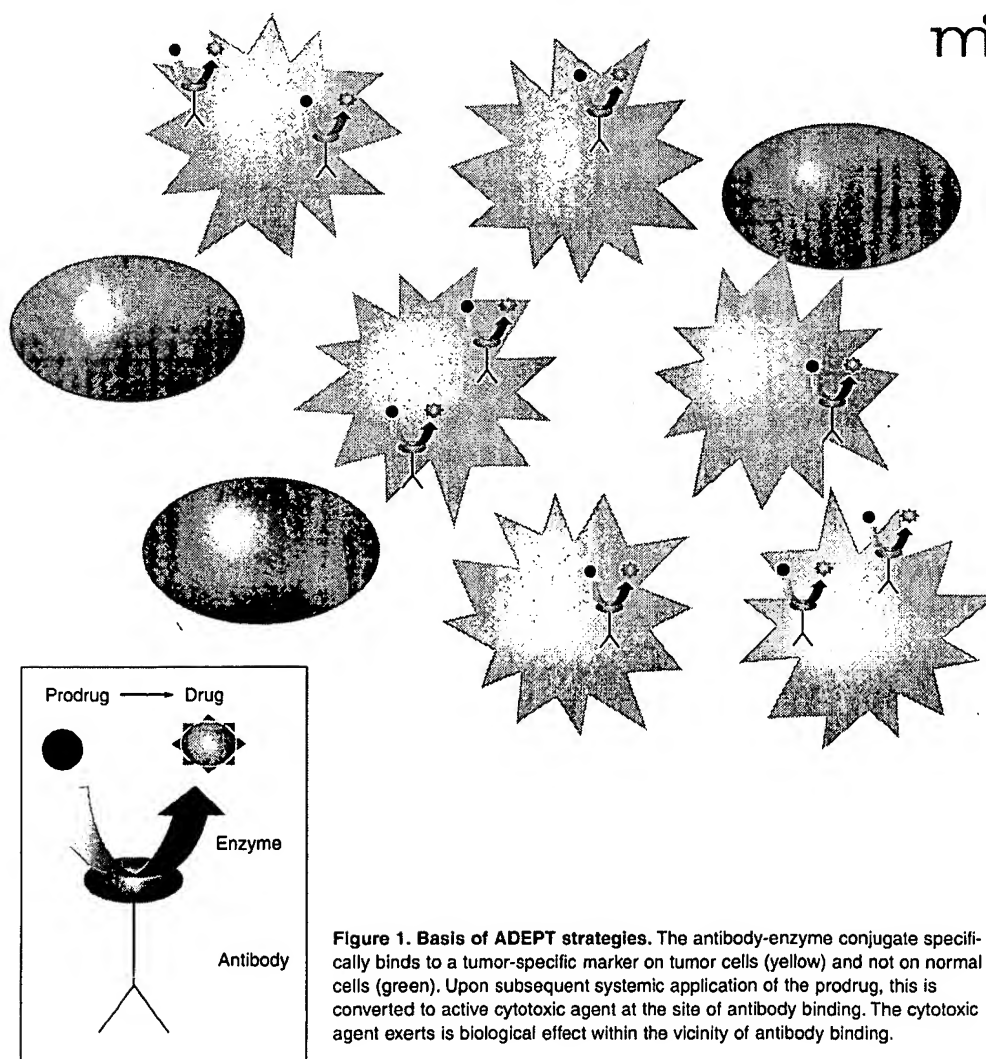


Figure 1. Basis of ADEPT strategies. The antibody-enzyme conjugate specifically binds to a tumor-specific marker on tumor cells (yellow) and not on normal cells (green). Upon subsequent systemic application of the prodrug, this is converted to active cytotoxic agent at the site of antibody binding. The cytotoxic agent exerts its biological effect within the vicinity of antibody binding.

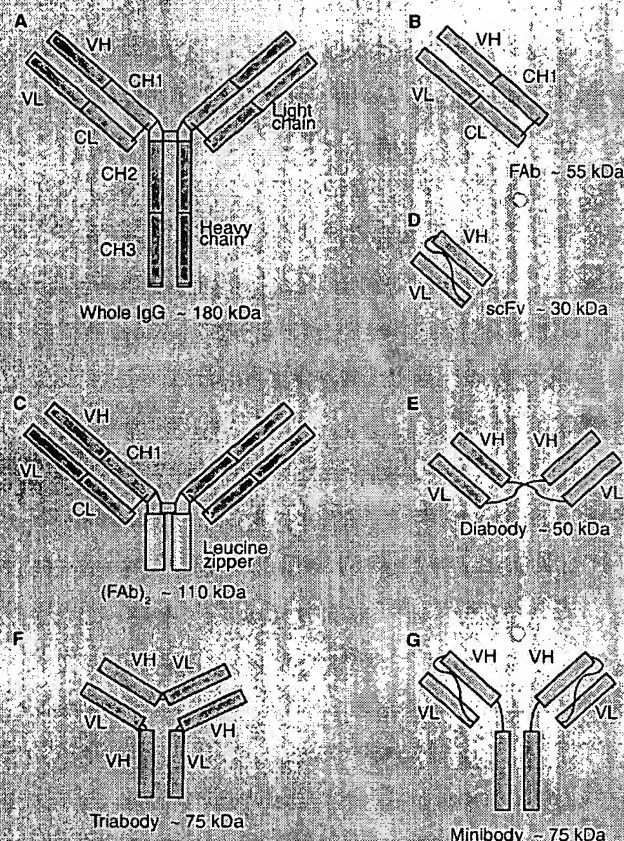
Box 1. Recombinant Antibody Fragments

The IgG antibody (**A**) consists of a heavy and a light chain (H and L), both of which contain variable (V) and constant regions (C). The binding pocket of the antibody that recognizes its specific epitope consists of the variable regions of both the heavy and light chains (V_H and V_L), and only this region is required to produce a molecule capable of binding.

Fab fragments can be produced by proteolytic cleavage of a monoclonal antibody, or by recombinant engineering, to remove the Fc domain, and can be of the form Fab or $(Fab)_2$. The Fab fragments include the complete light chain of the antibody linked by a disulphide bond to the V_H - CH_1 fragment of the heavy chain (**B**). The production of homospecific or bispecific $(Fab)_2$ dimeric antibodies is possible, and dimer association can be strengthened by inclusion of a genetically engineered leucine zipper (**C**).

By using recombinant technology, it is possible to generate a variety of smaller fragments of the IgG molecule that retain or have improved binding affinities.

One of the most common forms is the single chain variable fragment (scFv), produced by combination of V_H and V_L joined by a flexible peptide linker (**D**). In the scFv molecule, the V_L and V_H domains can freely associate in an intramolecular relationship. Shortening of the linker length can inhibit this intramolecular interaction, and intermolecular association of V_L and V_H domains becomes more energetically favorable, resulting in the formation of diabodies (**E**), triabodies (**F**) or even tetrabodies. The scFv form has been further modified to include constant domains such as CH_3 for the creation of minibodies (**G**), which are composed



of either homo- or hetero-dimers of the scFv- CH_3 . Minibodies have the advantage of increased stability and bivalent specificities if required.

antibody and demonstrated therapeutic indices unattainable with unconjugated cytokine. The anti-tumor activity of this experimental therapeutic agent is enhanced synergistically when used in combination with an L19-IL-2 fusion (20). Both these molecules are now in early stage clinical evaluation.

ADEPT and immunocytokines are only two of a range of immunotherapeutic protein or peptide strategies currently in pre-clinical and clinical development for a range of diseases, predominantly in the field of oncology. Any peptide or protein shown to have therapeutic effect can be exploited potentially in the form of an immunoconjugate for the treatment of a plethora of disease conditions. The range of possibilities will ensure continued interest in this area and marketing of eventual drugs in this class.

DRUG-IMMUNOCONJUGATES

Conventional cancer treatment frequently involves administering a regime of chemotherapeutic drugs that may be used as the primary means of intervention or as an adjuvant to surgical removal of the tumor mass. Systemic administration of chemotherapeutic drugs results in the death of actively dividing cells within the body, specifically cancer cells, but also has the undesired side effects of destroying oral and intestinal mucosa, hair follicles, and bone marrow. Much attention has been directed to selective targeting of these agents by conjugation to antibodies against tumor specific markers, in an attempt to improve efficacy and reduce side effects.

As early as the mid-1970s, antibody-drug conjugates were being investigated as an approach for targeting chemotherapeutic drugs to tumor cells (21). In these early investigations, drugs such as methotrexate were covalently linked to human serum albumin as

a carrier, which in turn could then be chemically linked to a monoclonal antibody. Garnett and coworkers compared the use of this approach for targeted cytotoxicity with that of conventional systemic delivery toward an osteogenic sarcoma cell line. The conjugate was selective in its action and was preferentially cytotoxic towards antibody-reactive cell types under a competitive assay with non-conjugated antibody (22).

In 2000, the FDA granted approval for the first antibody drug conjugate for human therapeutic use. Humanized CD33-specific MAb conjugate [gemtuzumab ozogamicin (Mylotarg[®])] was licensed for the treatment of acute myeloid leukemia (AML) in patients over sixty years of age and deemed unsuitable for other chemotherapy (23). This "first-in-class" therapeutic consists of gemtuzumab, a labile linker attached to Lys side-chains, and then conjugated to a calicheamicin hydrazide derivative. Approximately two or three molecules of calicheamicin are attached per antibody, and the therapeutic formulation consists of 50% of the antibody labeled by the drug. Mylotarg[®] binds to the cell surface marker CD33, a receptor overexpressed on acute myeloid leukemia cells and on normal leukocytes and leukemic progenitors, but which is absent on normal stem cells. Once Mylotarg[®] binds to CD33, the receptor-antibody conjugate complex is internalized and hydrolyzed. The resulting hydrolysis releases calicheamicin, which freely localizes in the nucleus, initiating DNA alkylation and promoting cell death (24, 25). In preclinical models, Mylotarg[®] selectively and potently inhibited CD33⁺ AML cell lines, and clinical studies have revealed complete remission in up to 30% of adults with relapsed CD33⁺ AML (26).

In addition to the selection of a suitable chemotherapeutic agent and exploitation of a highly specific antibody towards a biomarker of myeloid leukemia, the efficacy of Mylotarg[®] is, in large part, because of the linker chemistry between antibody and drug. The linker region is crucial in these molecules to ensure that the hybrid molecule: 1) is stable, 2) can successfully deliver the drug to the target cell, tissue or organ, and 3) can present the drug appropriately to permit therapeutic action. The most popular approach for the development of immunochemotherapeutics is to use an

antibody that upon binding to its specific cell surface biomarker will internalize; a mechanism referred to as receptor-mediated endocytosis. Endocytosis will direct the immunochemotherapeutic agent to endosomes, where fusion with activated lysosomes initiates metabolism and degradation of the endosome contents. The nature and composition of the activated lysosomes offer many possibilities to the drug developers for the specific release of the drug compound. In the case of Mylotarg[®], a hybrid linker is used that contains both a hydrazone and sterically-confined disulfide, which are labile only in acid (hydrazone) and reductive (disulfide) environments. In the activated lysosomes, the acidic conditions (pH 5) and high glutathione concentrations can cleave the hydrazone and reduce the disulfide, resulting in delayed release of the drug from its delivery antibody after endocytosis. In addition to the hydrazone and disulfide linkers (as used in Mylotarg[®]), other linker chemistries are being examined. One of the most promising strategies involves the use of protease-specific peptide sequences, which can be cleaved by activated proteases, such as the lysosomal cysteine protease cathepsin B (27). Depending on the nature of the drug or toxin, protease recognition sequences can be created through genetic engineering or by peptide chemistry. Using this strategy, Dubowchik and colleagues created protease-sensitive sequences in conjugates and demonstrated improved stability and specificity as compared to those observed in hydrazone conjugates (28). In these studies, the investigators found that dipeptide sequences that are sensitive to proteolytic cleavage by cathepsin B (e.g., phenylalanine-lysine and valine-citrulline) were effectively hydrolyzed by the protease when used as a linker between antibody-doxorubicin conjugate, after internalization of the conjugate-antigen complex.

In addition to calicheamicin, doxorubicin represents one of the most commonly researched drugs in antibody-based applications. Doxorubicin, an anthracycline, produces its cytotoxic effects by alkylation of double-stranded DNA, resulting in interstrand cross-linking, triggering apoptosis (29). Tolcher and coworkers conjugated this chemotherapeutic agent (via a hydrazone linker) to a chimeric antibody (BR96) that targets Lewis(y), a tetrasaccharide overexpressed on the surface of many tumors, and in 75% of breast carcinomas (30). Most normal tissues, except for epithelial cells in the GI tract, do not express this tumor antigen (31). Although BR96-doxorubicin conjugate demonstrated efficacy against metastatic breast and non-small cell lung tumors, Phase I and II clinical trials also highlighted significant gastro-intestinal toxicity due to the presence of the antigen in the GI tract; it is now being evaluated under reduced dosing in combination therapy with docetaxel (32).

A further drug family that has been used in antibody-targeted delivery are the auristatins, based on the naturally occurring peptide termed Dolastatin 10. The auristatins are highly potent peptides that inhibit tubulin polymerization, producing antimitotic effects that can be exploited therapeutically (33). Synthetic auristatin analogs such as monomethyl auristatin (MMAE) and auristatin E (AE) have been conjugated to tumor-specific antibodies. Senter and colleagues succeeded in producing highly efficacious molecules that targeted

Table 2. Common Alpha and Beta Radionuclides Used for Radioimmunotherapy

Radionuclide	Emission	Half-Life	Max Tissue Penetration (mm)
⁹⁰ Y	β	2.7 days	12
¹³¹ I	β	8 days	2
¹⁷⁷ Lu	β	6.7 days	1.5
¹⁸⁶ Re	β	3.8 days	5
¹⁸⁸ Re	β	0.7 days	11
²¹² Bi	α	60 min	0.6–0.9
²¹³ Bi	α	46 min	0.1
²²⁵ Ac	α	10 days	0.04
²¹¹ At	α	7 hrs	0.05

carcinomas and hematologic malignancies [using Lewis(y)- and CD20-specific antibodies, respectively] and were effective at one-sixtieth of the maximum tolerated dose (34).

In addition to these chemotherapeutic agents, protein-based toxins have also been examined in their potential as immunotherapeutics. Using recombinant antibody technology it is possible to express antibodies or antibody fragments with a toxic fusion protein partner that can be cleaved upon internalization by proteases such as cathepsin B or E. Protein toxins that have been conjugated or fused to delivery antibodies include ricin, gelonin, *Pseudomonas* exotoxin, diphtheria toxin, and RNase (35). Gelonin consists of a single polypeptide and is a ribosome-inactivating toxin with a biological activity similar to that of ricin, a potent inhibitor of protein synthesis. The humanized CD33-specific MAb HuM195 linked to recombinant gelonin exhibits much more potency than the non-fused MAb when applied administered in vitro to primary blast cultures from patients with AML (36). Development of this and other immunotoxins as effective therapeutics represents a promising area of research, especially when one considers the wide range of toxic proteins or molecules that can be conjugated to a targeting antibody. The main challenges for this type of immunoconjugate are preventing or limiting toxic side effects and rapid clearance problems owing to the inherent immunogenicity of the toxins, which are almost entirely nonhuman in nature.

RADIOIMMUNOCONJUGATES

The use of nuclear medicine, both in diagnostics and therapeutics relies implicitly on accurate delivery to the disease location. Within this field, the application of antibodies as delivery agents for the radioisotopes holds much promise, as two of the three currently licensed antibody conjugates at this time are radioimmunoconjugates. As with other immunoconjugate strategies, confirmation that a radioisotope can be stably conjugated to an antibody without adversely affecting its biological activity has led to their widespread evaluation in both the imaging (immunoscinigraphy) and radioimmunotherapy (RIT). Depending on the nature of the radioligand used, the same antibody can be used to target specifically the site of disease for either toxic effector function, or imaging and diagnosis.

The FDA has approved two radiolabeled antibodies—ibritumomab tiuxetan and tositumomab—both of which target CD20, a cell surface marker expressed on over 95% of B cells and B cell lymphoma. CD20 is not expressed on stem cells or on terminal differentiated plasma cells; therefore, the removal of CD20⁺ cells from the patient has no long-term effects. CD20-targeted RIT has been successful because lymphoma cells are very accessible and allow rapid binding of MAb. The disease characteristics also allow delivery of high energy particles to the tumor cells.

The ⁹⁰Y-conjugated ibritumomab tiuxetan (Zevalin[®]) relies on the covalent conjugation of a metal chelating agent, tiuxetan, to a chimeric IgG₁ kappa antibody (37–39). The tiuxetan group chelates the ⁹⁰Y metal isotope for use as a therapy or, alternatively, the

gamma-emitter ¹¹¹In for use in imaging. Tositumomab (Bexxar[®]), consists of a CD20-specific antibody conjugated to ¹³¹I, using direct oxidant-based chemistry, before chromatographic purification (40).

Both approved antibodies (Bexxar[®] and Zevalin[®]) employ beta-emitting radioisotopes that have therapeutic applications in the treatment of hematological malignancies. Beta emitters induce cell death by forming free radicals in the target cell. The beta emissions of ⁹⁰Y, with a half-life of sixty-four hours (Table 2), deposit energy over a relatively large area (i.e., several mm), leading to possible dissipation into neighboring cells but less likely to cause widespread damage to surrounding normal tissue. Novel radiolabels, however, with more favorable linear energy transfer, half-life decay, and path length characteristics are in development. Alpha emitters (i.e., ²¹¹At) are presently being considered for therapeutic use because of their high ionization potential per unit path length. They are more effective at selectively killing target cells and lack the ability to penetrate adjacent cells (41). The careful selection and matching of differing energy-depositing radionuclides to disease lesions, depending on type and bulk of tumor tissue, will facilitate improved efficacies to these radioimmunoconjugates (42).

The challenges faced by radioisotope delivery are similar to those faced by other modes of immunotherapy, namely specific localization, clearance and system toxicity. One methodology being examined is a pre-targeting approach using bispecific antibodies (43). The development of recombinant antibodies with dual specificity (bispecific) toward a tumor-associated target and toward a functional moiety such as a metal chelating group (for radionuclide binding) has introduced the concept of “pretargeting” for certain diseases. In this therapeutic approach, the patient is given a suitable bispecific antibody, which is given sufficient time to localize to the disease site, while the excess remainder of the antibody is cleared. The systemic application of a suitable radionuclide hapten follows, which then binds to the other binding site on the antibody, thereby allowing localization of the radionuclide to the site of disease to exert its cytotoxic response (44). Other pretargeting approaches have exploited streptavidin-biotin chemistry (45) and complementary oligonucleotide approaches (46) in order to home the targeted radionuclide to the site of bispecific antibody binding. The pretargeting approach allows improved flexibility in the nature of the radionuclide used for both therapy and imaging. Recent advances in availability of various radionuclides and in antibody engineering abilities have injected interest and optimism in this, and other radioimmunoconjugate methodologies.

The use of radiolabeled recombinant antibody fragments has also aided the specific targeting of tumors with more favorable clearance profiles and less non specific binding, for example by attenuation of Fc-FcRn (neonatal Fc receptor) interactions (47). Biodistribution studies have shown that engineered antibody fragments such as single chain Fv (scFvs), diabodies, and minibodies have superior uptake from the blood, particularly useful for imaging (48) and, in some cases, they exhibit favorable tumor localization and penetration (49) (Box 1).

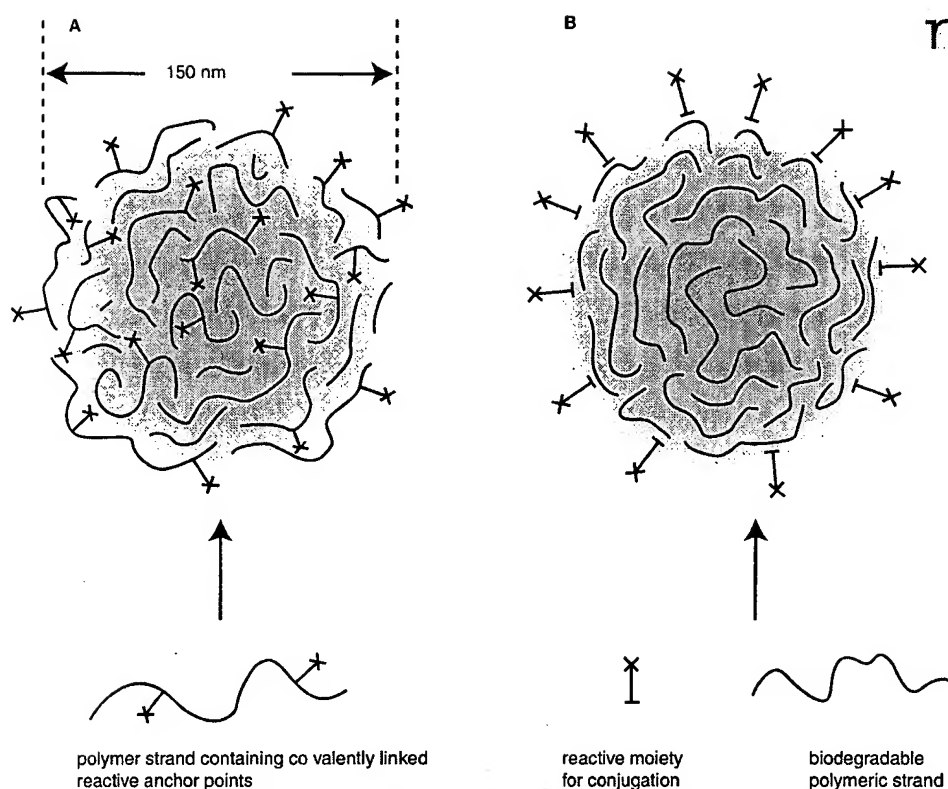


Figure 2. A schematic representation of polymeric nanoparticles of approximately 150 nm diameter with anchor points for conjugation. These anchors can form an integral and covalent part of the polymer stand prior to nanoparticle assembly (A). PLGA, commonly used to make solid nanoparticles, lacks the necessary functional groups for covalent conjugation. Functionality added prior to assembly ensures that at least some of the functional groups exist at the surface, with most submerged within the nanoparticle matrix. Alternatively, functionality can be adsorbed onto the nanoparticle surface once its assembly is complete (B).

Conventional whole IgG radiolabeled MAbs exhibit impaired killing efficacy arising from the short retention time of the radionuclide within the targeted cell. The problem of retention time within target cells can be addressed by "residualizing" the radiolabels. The principle of the technique involves the inclusion of a radionuclide as a component of a non-metabolizable peptide (for example, diethylenetriaminepentaacetic acid-appended radioiodinated peptides that contain D-amino acids) designed to become internalized by receptor-mediated endocytosis and localized in the lysosome following antibody catabolism (50). Once trapped, the radiolabel is capable of causing maximum effect.

As with all antibody-based therapeutic approaches, the development of accompanying diagnostic tools to the radioimmunoconjugates is as important as the therapeutic agent itself. In this area, the emerging techniques for the application of radiolabeled antibodies in whole-body or tumor imaging is particularly exciting, providing data on tumor localization and metastases as well as insight into biodistribution patterns of potential medications. Positron emission tomography (PET) is the four dimensional imaging of a radiotracer (e.g., ^{18}F , ^{64}Cu , ^{86}Y , ^{76}Br , or ^{124}I) within a living system (51). The diagnostic potential of positron emitter-coupled antibod-

ies has suffered because of the short half-life of radiotracers and the slow systemic clearance of antibodies. Recent evidence has demonstrated that the positron emitter ^{89}Zr , which has a kinetic profile that matches that of whole antibodies, can act as a reliable predictor of therapeutic antibody biodistribution (52). Advances in antibody engineering to improve the clearance and localization kinetics of the labeled antibodies will further enhance their use, and the use of more stable conjugation chemistries [for example, sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate] will contribute to the growing application and success of radioimmunoconjugates.

IMMUNOCOLLOIDAL CONJUGATES

The application of colloids, such as nanoparticles and liposomes, has become an important focus within drug

delivery and targeting. The small size of colloids allows for their passage through the narrowest of capillaries and endothelial spaces, delivering a wide range of drugs to the body for sustained periods of time. In addition, they form a drug delivery vector with much adaptability, making them a generic system that can be tailored to carry several types of drug substance and have a conjugated targeting system decorated upon their outer surface.

Targeted drug delivery using colloidal systems can be achieved by either passive or active targeting. The former refers to the accumulation of a carrier system at distinct sites dictated by factors of physicochemical or physiological means (53). This is exemplified by selective nanoparticulate uptake at sites of inflamed colonic mucosa. Similarly, the enhanced permeation and retention effect permits colloidal nanoparticles to accumulate in tumor tissue compartments (54), which is driven by the vascular endothelium of angiogenic blood vessels having gaps (600–800 nm) between adjacent endothelial cells that exceed the nanoparticle diameter (100–500 nm). Also, nanoparticles with hydrophobic surfaces demonstrate increased uptake by the mononuclear phagocytic system and may be a useful strategy to treat diseases of reticuloendothelial organs. Active targeting, on the other hand, requires modification of the naked nanopar-

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ticle, usually by conjugation with a recognizing moiety, such as a specific and selective antibody to facilitate targeting (55).

To achieve antibody-mediated targeting, peripheral attachment of the antibody to the colloid is needed. This is generally a two-step procedure where the nanoparticle surface is primed with a suitable functional group prior to the second step involving ligand attachment. One method of nanoparticle priming can be achieved using a formulation with modified polymeric strands (Figure 2A), such as those based on poly(lactide-co-glycolide) acid (PLGA). The use of modified strands ensures that the resulting copolymer retains some biodegradability and biocompatibility, a property that is conferred to the primed nanoparticle.

Amine functionality, for example, can be added by copolymerization with poly(lysine) (56) or by using newly synthesized polymers, such as PEG-NH₂ (Figure 3). Further modification is possible, where block copolymers comprising poly(lactic acid)-poly(ethylene glycol)-biotin

(PLA-PEG-biotin) have been described and used to assemble nanoparticulate systems (57) (Figure 3B). This modification exploits avidin-biotin binding and is a novel method for noncovalent bonding of an avidin-containing ligand to a biotin-presenting nanoparticulate surface. A simpler method for introducing functionality to a nanoparticle periphery is to use surface adsorption without any form of covalent chemistry (Figure 2B). Most nanoparticle suspensions require a surface-bound stabilizer such as a nonionic surfactant to prevent coalescence. Replacing some of this stabilizer with poly(ethylene-alt-maleic acid) has been used to introduce carboxylic groupings to the nanoparticle surface (58). Similarly, adsorption of avidin-palmitic acid has been used to generate an avidin-rich surface. These approaches may suffer drawbacks, such as difficulty in preparation and loss of the targeting effect that is exacerbated with time owing to desorption or degradation of adsorbed groups as the nanoparticle scaffold erodes.

Liposomes are colloidal drug carriers, of similar dimension to nanoparticles, but consisting instead of one or more phospholipid bilayers enclosing an aqueous core. They take on the form of large multilamellar (MLV), small unilamellar (SUV), or large unilamellar

(LUV) vesicles. Water-soluble drugs occupy the aqueous compartments, whereas those of more lipophilic character occupy the lipid bilayers. Drug targeting with liposomes has been discussed in depth elsewhere, whereas the term "immunoliposome" has been coined to describe a liposome conjugated to an antibody or portions of antibody (59). Similar chemistries to those used in nanoparticle conjugation have been utilized with liposomes, all aiming to attach an antibody or ligand to the outer shell of the liposome. Biotinylated phospholipids and avidin have been used in the liposomal formation, which can then be used to bind biotinylated antibodies. Covalent attachment of a thiolated antibody through a thioether bond formed with the maleimide group in maleimide-derivatized phospholipids has been described. Similar covalent attachments using carbodiimide between amine groups of the phospholipid or maleimidated phosphatidylethanolamine and carboxyl moieties of antibody have been studied (60).

Various studies have demonstrated the potential for antibody-conjugated colloidal systems, of both solid nanoparticle and liposomal formulation. These include the application of biotinylated CD3-specific antibodies—which recognize T-cell leukemia cells and primary T-lymphocytes—that were conjugated to thiolated gelatin nanoparticles through neutravidin-biotin noncovalent bonds. In vitro studies showed a selective uptake (by receptor mediated endocytosis) of these conjugated nanoparticles in 84% of a T-lymphocyte

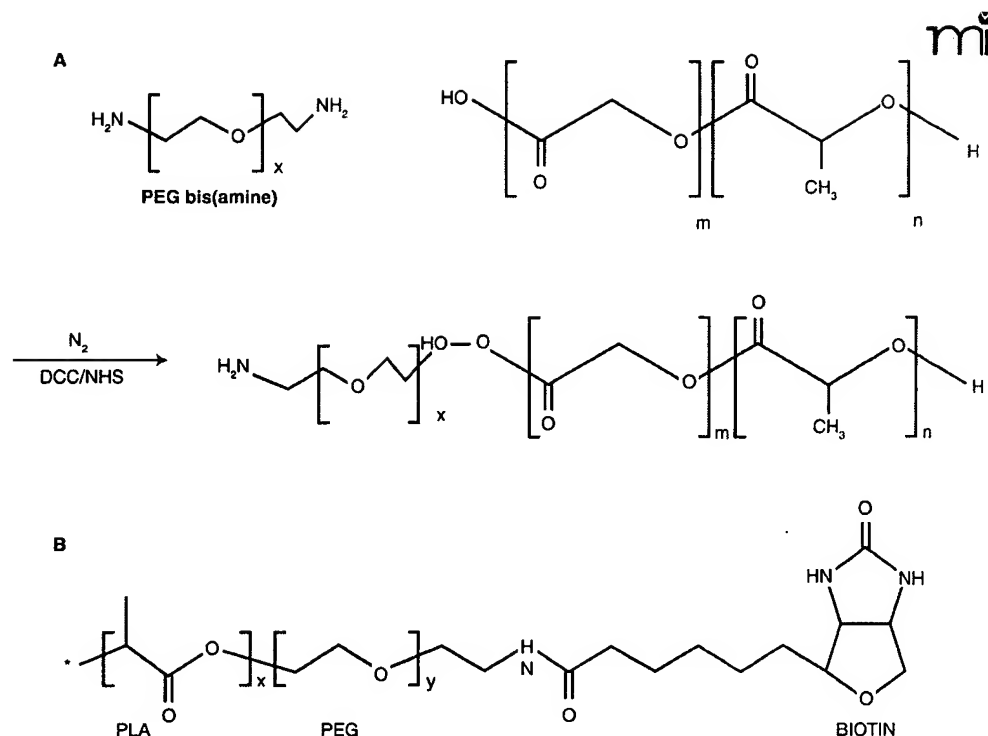


Figure 3. Chemical structures of molecules used in linking antibodies to active group moieties. A. Copolymerization of poly(ethylene glycol)-bis(amine) with poly(lactide-co-glycolide) to give a polymeric strand presenting a terminal end amine group. **B.** Further attachment of a biotin group gives a polymeric structure that can be formed into nanoparticles that presents an attachment point for avidin-containing ligands.

population. PEG-coated liposomes conjugated with folate on their outer surfaces were targeted to folate receptor-expressing tumors. In vitro results demonstrated enhanced cytotoxicity of encapsulated liposomal drugs through endocytotic liposomal drug uptake, whereas in vivo results have remained less encouraging (61). Wheat germ agglutinin has been conjugated to PLGA nanoparticles loaded with paclitaxel for localized pulmonary delivery. In vitro results showed that these nanoparticles exhibited enhanced cytotoxicity through improved cellular uptake through wheat germ agglutinin receptor-mediated endocytosis (62). Similarly, a Her2 receptor-specific antibody (trastuzumab) was conjugated to the surface of gelatin-human serum albumin nanoparticles through avidin-biotin non-covalent bonding. In vitro results showed effective internalization of the nanoparticles by Her2-overexpressing tumor cells via receptor-mediated endocytosis. A selectin ligand [Sialyl-Lewis (SLe^x)]- and ICAM-1-specific antibody recognizing endothelial-expressed inflammation markers were conjugated to PLGA microspheres and acted as leukocyte mimetics for targeting drugs to the vasculature in inflammatory diseases (63). OX26 MAbs were conjugated to the surface of liposomes that contained daunomycin and were used to bypass P-glycoprotein-mediated effects in multi-drug resistant RBE4 cells. Subsequent studies revealed brain accumulation of daunomycin from OX26 immunoliposomes to be higher as compared to brain accumulation of free (unconjugated) drug (64). Additionally, immunoliposomes containing doxorubicin conjugated with scFv fragment A5 (scFv A5) directed against human endoglin showed an increase in cytotoxicity towards endothelial cells compared to non-targeted liposomes and free drug in vitro (65).

Clearly, these examples show there is a verifiably enhanced cellular response brought about by targeted colloidal delivery, albeit during in vitro experimentation. The challenge now remains for these findings to be applied successfully to clinical situations such as those demanding specific delivery of cytotoxic agents to multifocal sites of neoplastic growth.

PERSPECTIVE

The advantages to be gained from the targeting of drug substances to specific cellular targets are apparent. However, the need to advance from drug-based therapies that rely primarily on pharmacodynamic and pharmacokinetic drivers to dictate drug distribution is pressing, certainly within the field of oncology. The difficulties encountered in current chemotherapy of neoplastic disease, such as the emergence of dose-limiting side effects, support this contention. Moving away from drug distribution dictated by physicochemical considerations (e.g., bioavailability and drug solubility) has been a difficult objective to achieve. Innovative strategies that exploit the innate ability of antibodies to attach to an antigen hold most promise in achieving this. The challenge remains that an antibody-conjugate assembled in such a way to transport a therapeutic payload to a cellular site must meet demanding criteria, including specificity, safety, and stability. Already, conjugates based on protein-antibody

and antibody-colloid constructs have been described. The full translation of these technologies to the clinical environment remains the next big challenge. doi:10.1124/mi.5.6.9

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References

- Kim, S.J., Park, Y., and Hong, H.J. Antibody engineering for the development of therapeutic antibodies. *Mol. Cells* 20, 17–29 (2005).
- Kohler, G. and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256, 495–497 (1974).
- Shawler D.L., Bartholomew R.M., Smith L. M., and Dillman R.O. Human immune response to multiple injections of murine monoclonal IgG. *J. Immunol.* 135, 1530–1535 (1985).
- Baker, M. Upping the ante on antibodies. *Nat. Biotechnol.* 23, 1065–1072 (2005).
- Steplewski, Z., Lubeck, M.D., and Koprowski, H. Human macrophages armed with murine immunoglobulin G2a antibodies to tumors destroy human cancer cells. *Science* 221, 865–867 (1983).
- Adams, G.P. and Weiner, L.M. Monoclonal antibody therapy of cancer. *Nat. Biotechnol.* 23, 1147–1157 (2005). An up-to-date review on all aspects of antibody therapeutic development for the treatment of cancer.
- Di Gaetano, N., Cittera, E., Nota, R., Vecchi, A., Grieco, V., Scanziani, E., Botto, M., Introna, M., and Golay, J. Complement activation determines the therapeutic activity of rituximab in vivo. *J. Immunol.* 171, 1581–1587 (2003).
- Senter, P.D. and Springer, C.J. Selective activation of anti-cancer prodrugs by monoclonal antibody-enzyme conjugates. *Adv. Drug Deliv. Rev.* 53, 247–264 (2001).
- Wu, A.M. and Senter, P.D. Arming antibodies: Prospects and challenges for immunoconjugates. *Nat. Biotechnol.* 23, 1137–1146 (2005).
- Meyer, D.L., Jungheim, L.N., Mikolajczyk, S.D., Shepherd, T.A., Starling, J.J., and Ahlem, C.N. Preparation and characterization of a beta-lactamase-Fab' conjugate for the site-specific activation of oncolytic agents. *Bioconjug. Chem.* 3, 42–48 (1992).
- Mikolajczyk, S.D., Meyer, D.L., Starling, J.J., Law, K.L., Rose, K., Dufour, B., and Offord, R.E. High yield, site-specific coupling of N-terminally modified beta-lactamase to a proteolytically derived single-sulfhydryl murine Fab'. *Bioconjug. Chem.* 5, 636–646 (1994).
- Schnee, J.M., Runge, M.S., Matsueda, G.R., Hudson, N.W., Seidman, J.G., Haber, E., and Quenberger, T. Construction and expression of a recombinant antibody-targeted plasminogen activator. *Proc. Natl. Acad. Sci. U.S.A.*, 84, 6904–6908 (1987).
- Gillies, S.D., Young, D., Lo, K.M., Foley, S.F., and Reisfeld, R.A. Expression of genetically engineered immunoconjugates of lymphocytes and a chimeric anti-ganglioside GD2 antibody. *Hybridoma* 10, 347–356 (1991).
- Bagshawe, K.D., Springer, C.J., Searle, F., Antoniow, P., Sharma, S.K., Melton, R.G., and Sherwood, R.F. A cytotoxic agent can be generated selectively at cancer sites. *Br. J. Cancer* 58, 700–703 (1988). Landmark publication in the field of ADEPT methodology.
- Neri, D., Carnemolla, B., Nissim, A. et al. Targeting by affinity-matured recombinant antibody fragments of an angiogenesis associated fibronectin isoform. *Nat. Biotechnol.* 15, 1271–1275 (1997).
- Rooseboom, M., Commandeur, J.N.M., Vermeulen, N.P.E. Enzyme-catalysed activation of anticancer drugs. *Pharm. Rev.* 56, 53–102 (2004).

Detailed review of strategies for catalytic activation of pro-drugs.

17. Lode, H.N., Xiang, R., Becker, J.C., Gillies, S.D., and Reisfeld, R.A. Immunocytokines: a promising approach to cancer immunotherapy. *Pharmacol. Ther.* 80, 277–292 (1998).
18. Becker, J.C., Pancook, J.D., Gillies, S.D., Mendelsohn, J., and Reisfeld, R.A. Eradication of human hepatic and pulmonary melanoma metastases in SCID mice by antibody-interleukin 2 fusion proteins. *Proc. Natl. Acad. Sci. U.S.A.*, 93, 2702–2707 (1996).
19. Choi, B.S., Sondel, P.M., Hank, J.A. et al. Phase I trial of combined treatment with ch14.18 and R24 monoclonal antibodies and interleukin-2 for patients with melanoma or sarcoma. *Cancer Immunol Immunother.* Epub ahead of print DOI: 10.1007/s00262-005-0069-7 (2005).
20. Borsi, L., Balza, E., Carnemolla, B. et al. Selective targeted delivery of TNF α to tumor blood vessels. *Blood* 102, 4384–4392 (2003).
21. Rowland, G.F., O'Neill, G.J., and Davies, D.A. Suppression of tumor growth in mice by a drug-antibody conjugate using a novel approach to linkage. *Nature* 255, 487–488 (1975). **Landmark paper describing use of a linker for conjugation between antibody and drug to overcome stability and dissociation problems observed with antibodies directly conjugated to drugs.**
22. Garnett, M.C., Embleton, M.J., Jacobs, E., and Baldwin, R.W. Preparation and properties of a drug-carrier-antibody conjugate showing selective antibody-directed cytotoxicity in vitro. *Int. J. Cancer* 31, 661–670 (1983).
23. Sorokin, P. Mylotarg approved for patients with CD33+ acute myeloid leukemia. *Clin. J. Oncol. Nurs.* 4, 279–280 (2000). **A short article relating the approval and administration of the first antibody-drug conjugate for human therapeutic use.**
24. Voutsadakis, I.A. Gemtuzumab ozogamicin (CMA-676, Mylotarg) for the treatment of CD33+ acute myeloid leukemia. *Anticancer Drugs* 13, 685–692 (2002).
25. Feldman, E.J. Monoclonal antibody therapy in acute myeloid leukemia. *Curr. Hematol. Rep.* 2, 73–77 (2003).
26. Linenberger, M.L. CD33-directed therapy with gemtuzumab ozogamicin in acute myeloid leukaemia: progress in understanding cytotoxicity and potential mechanisms of drug resistance. *Leukemia* 19, 176–182 (2005).
27. Dubowchik, G.M. and Firestone, R.A. Cathepsin B-sensitive dipeptide prodrugs. 1. A model study of structural requirements for efficient release of doxorubicin. *Bioorg. Med. Chem. Lett.* 8, 3341–3346 (1998).
28. Dubowchik, G.M., Firestone, R.A., Padilla, L., Willner, D., Hofstead, S.J., Mosure, K., Knipe, J.O., Lasch, S.J., and Trail, P.A. Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: model studies of enzymatic drug release and antigen-specific in vitro anticancer activity. *Bioconjug. Chem.* 13, 855–869 (2002).
29. Zwelling, L.A., Altschuler, E., Cherif, A., and Farquhar, D. N-(5,5 diacet oxypentyl)doxorubicin: a novel anthracycline producing DNA interstrand cross-linking and rapid endonucleolytic cleavage in human leukemia cells. *Cancer Res.* 51, 6704–6707 (1991). **This paper describes the first of a family of alkylating anthracyclines designed to be water soluble, easy to formulate, and capable of producing DNA interstrand cross-linking.**
30. Tolcher, A.W., Sugarman, S., Gelmon, K.A. et al. Randomized phase II study of BR96-doxorubicin conjugate in patients with metastatic breast cancer. *J. Clin. Oncol.* 17, 478–484 (1999).
31. Hellstrom, I., Garrigues, H.J., Garrigues, U., and Hellstrom, K.E. Highly tumor-reactive, internalizing, mouse monoclonal antibodies to Le(Y)-related cell surface antigens. *Cancer Res.* 50, 2183–2190 (1990).
32. Ross, H.J., Rudin, C.M., Hart, L.L., Swanson, P.M., Rarick, M.U., Figlin, R.A., Jacobs, A.D., and Miller, D.M. Randomized phase II study of SGN-15 (cBR96-doxorubicin immunoconjugate) with docitaxel in patients with advanced or metastatic non-small cell lung cancer (NSCLC) [Abstract no. 7039]. *J. Clin. Oncol.* 22, 626s (2004).
33. Madden, T., Tran, H.T., Beck, D., Huie, R., Newman, R.A., Pusztal, L., Wright, J.J., and Abbruzzese, J.L. Novel marine-derived anticancer agents: A phase I clinical, pharmacological, and pharmacodynamic study of dolastatin 10 (NSC 376128) in patients with advanced solid tumors. *Clin. Cancer Res.* 6, 1293–1301 (2000).
34. Doronina, S.O., Toki, B.E., Torgov, M.Y. et al. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol.* 21, 778–784 (2003).
35. Govindan, S.V., Griffiths, G.L., Hansen, H.J., Horak, I.D., and Goldenberg, D.M. Cancer therapy with radiolabeled and drug/toxin-conjugated antibodies. *Technol. Cancer Res. Treat.* 4, 375–391 (2005). **A comprehensive review on the current technologies for chemo- and radio-immunotherapy**
36. Pagliaro, L.C., Liu, B., Munker, R., Andreeff, M., Freireich, E.J., Scheinberg, D.A., Rosenblum, M.G. Humanized M195 monoclonal antibody conjugated to recombinant gelonin: An anti-CD33 immunotoxin with antileukemic activity. *Clin. Cancer Res.* 4, 1971–1976 (1998).
37. Fink-Bennett, D.M. and Thomas, K. 90Y-ibritumomab tiuxetan in the treatment of relapsed or refractory B-cell non-Hodgkin's lymphoma. *J. Nucl. Med. Technol.* 31, 61–68 (2003).
38. Krasner, C. and Joyce, R.M. Zevalin. 90Yttrium labeled anti-CD20 (ibritumomab tiuxetan), a new treatment for non-Hodgkin's lymphoma. *Curr. Pharm. Biotechnol.* 4, 341–349 (2001).
39. Tempero, M., Lechner, P., Baranowska-Kortylewicz, J., Harrison, K., Augustine, S., Schlom, J., Anderson, J., Wizecarver, J. and Colcher, D. High-dose therapy with 90yttrium-labeled monoclonal antibody CC49: A phase I trial. *Clin. Cancer Res.* 6, 3095–3102 (2000).
40. Wahl, R.L. Tositumomab and 131I therapy in non-Hodgkin's lymphoma. *J. Nucl. Med.* 46 Suppl 1, 128S–40S (2005).
41. Couturier, O., Supiot, S., Degraef-Mouglin, M., Faivre-Chauvet, A., Carlier, T., Chatal, J.F., Davodeau, F., and Cherel, M. Cancer radioimmunotherapy with alpha-emitting nuclides. *Eur. J. Nucl. Med. Mol. Imaging* 32, 601–614 (2005).
42. Wu, A.M. and Senter, P.D. Arming antibodies: prospects and challenges for immunoconjugates. *Nat. Biotechnol.* 23, 1137–1146 (2005).
43. Sharkey, R.M., Karacay, H., Cardillo, T.M., Chang, C.H., McBride, W.J., Rossi, E.A., Horak, I.D., and Goldenberg, D.M. Improving the delivery of radionuclides for imaging and therapy of cancer using pretargeting methods. *Clin. Cancer Res.* 11(19 Suppl), 7109s–7121s (2005). **This article details strategies for improving payload uptake by tumors using a pretargeting antibody, which has applications in both diagnosis and treatment.**
44. Karacay, H., McBride, W.J., Griffiths, G.L., Sharkey, R.M., Barbet, J., Hansen, H.J., and Goldenberg, D.M. Experimental pretargeting studies of cancer with a humanized anti-CEA x murine anti-[In-DTPA] bispecific antibody construct and a 99mTc-118Re-labeled peptide. *Bioconjug. Chem.* 11, 842–854 (2000).
45. Cremonesi, M., Ferrari, M., Chinol, M., Stabin, M.G., Grana, C., Prisco, G., Robertson, C., Tosi, G., and Paganelli, G. Three-step radioimmunotherapy with yttrium-90 biotin: Dosimetry and pharmacokinetics in cancer patients. *Eur. J. Nucl. Med.* 26, 110–120 (1999).
46. Liu, G., Mang'era, K., Liu, N., Gupta, S., Rusckowski, M., and Hnatowich, D.J. Tumor pretargeting in mice using 99mTc-labeled morpholino, a DNA analog. *J. Nucl. Med.* 43, 384–391 (2002).
47. Kenanova, V., Olafsen, T., Crow, D.M. et al. Tailoring the pharmacokinetics and positron emission tomography imaging properties of anti-carcinoma embryonic antigen single-chain Fv-Fc antibody fragments. *Cancer Res.* 65, 622–631 (2005). **This paper describes generating mutations in the FcRn receptor binding region of therapeutic antibodies to control the clearance kinetics of systemic antibody.**
48. Sundaresan, G., Yazaki, P.J., Shively, J.E. et al. 124I-labeled engineered anti-CEA minibodies and diabodies allow high-contrast, antigen-specific small-animal PET imaging of xenografts in athymic mice. *J. Nucl. Med.* 44, 1962–1969 (2003).
49. Adams, G.P., Schier, R., McCall, A.M., Simmons, H.H., Horak, E.M., Alpaugh, R.K., Marks, J.D., and Weiner, L.M. High affinity restricts the localization and tumor penetration of single-chain Fv antibody molecules. *Cancer Res.* 61, 4750–4755 (2001).
50. Stein, R., Govindan, S.V., Hayes, M., Griffiths, G.L., Hansen, H.J., Horak,

- I.D., and Goldenberg, D.M. Advantage of a residualizing iodine radiolabel in the therapy of a colon cancer xenograft targeted with an anticarcinoma embryonic antigen monoclonal antibody. *Clin. Cancer Res.* 11, 2727–2734 (2005). **This paper describes a method for trapping radiolabels in lysosomes, following antibody catabolism, by attachment of nonmetabolizable moieties thus facilitating effective tumor kill.**
51. Phelps, P. Positron emission tomography provides molecular imaging of biological processes. *Proc. Natl. Acad. Sci. U.S.A.* 97, 9226–9233 (2000).
 52. Verel, I., Visser, G.W., Boerman, O.C. et al. Long-lived positron emitters zirconium-89 and iodine-124 for scouting of therapeutic radioimmunoconjugates with PET. *Cancer Biother. Radiopharm.* 18, 655–661 (2003).
 53. Garnett, M.C. Targeted drug conjugates: principles and progress. *Adv. Drug Deliv. Rev.* 53, 171–216 (2001).
 54. Maeda, H. The enhanced permeability and retention (EPR) effect in tumor vasculature: The key role of tumor-selective macromolecular drug targeting. *Adv. Enzyme Regul.* 41, 189–207 (2001).
 55. Nishioka, Y. and Yoshino, H. Lymphatic targeting with nanoparticulate system. *Adv. Drug Deliv. Rev.* 47, 55–64 (2001).
 56. Caponetti, G., Hrkach, J.S., Kriwet, B., Poh, M., Lotan, N., Colombo, P., and Langer, R. Microparticles of novel branched copolymers of lactic acid and amino acids: Preparation and characterization. *J. Pharm. Sci.* 88, 136–141 (1999).
 57. Salem, A.K., Cannizzaro, S.M., Davies, M.C., Tendler, S.J., Roberts, C.J., Williams, P.M., and Shakesheff, K.M. Synthesis and characterization of a degradable poly(lactic acid)-poly(ethylene glycol) copolymer with biotinylated end groups. *Biomacromolecules* 2, 575–580 (2002).
 58. Keegan, M.E., Falcone, J.L., Leung, T.C., and Saltzman, W.M. Biodegradable microspheres with enhanced capacity for covalently bound surface ligands. *Macromolecules* 37, 9779–9784 (2004). **Describes a new method for presenting carboxylic acid moieties to the surface of nanoparticles, ideal for downstream conjugation of proteins.**
 59. Siwak, D.R., Tari, A.M., and Lopez-Berestein, G. The potential of drug-carrying immunoliposomes as anticancer agents. *Clin. Cancer Res.* 8, 955–956 (2002).
 60. Martin, F.J. and Papahadjopoulos, D. Irreversible coupling of immunoglobulin fragments to preformed vesicles. An improved method for liposome targeting. *J. Biol. Chem.* 257, 286–288 (1982).
 61. Gabizon, A., Shmeeda, H., Horowitz, A.T., and Zalipsky, S. Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid-PEG conjugates. *Adv. Drug Deliv. Rev.* 56, 1177–1192 (2004). **This paper describes the possibility of targeting tumor cells by using folic acid, which reacts to folate receptors on the surface of tumors.**
 62. Mo, Y. and Lim, L.Y. Preparation and in vitro anticancer activity of wheat germ agglutinin (WGA)-conjugated PLGA nanoparticles loaded with paclitaxel and isopropyl myristate. *J. Control Release* 107, 30–42 (2005). **This paper demonstrates the increase in cell cytotoxicity using paclitaxel loaded nanoparticles.**
 63. Omolola Eniola, A. and Hammer, D.A. In vitro characterization of leukocyte mimetic for targeting therapeutics to the endothelium using two receptors. *Biomaterials* 26, 7136–7144 (2005).
 64. Schnyder, A., Krahenbuhl, S., Drewe, J., and Huwyler, J. Targeting of daunomycin using biotinylated immunoliposomes: Pharmacokinetics, tissue distribution and in vitro pharmacological effects. *J. Drug Target.* 13, 325–335 (2005).
 65. Volkel, T., Holig, P., Merdan, T., Muller, R., and Kontermann, R.E. Targeting of immunoliposomes to endothelial cells using a single-chain Fv fragment directed against human endoglin (CD105). *Biochim. Biophys. Acta* 1663, 158–166 (2004).
 66. Senter, P.D., Saulnier, M.G., Schreiber, G.J., Hirschberg, D.L., Brown, J.P., Hellstrom, I., and Hellstrom, K.E. Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate. *Proc. Natl. Acad. Sci. U.S.A.* 85, 4842–4846 (1988).
 67. Senter, P.D. Activation of prodrugs by antibody-enzyme conjugates: A new approach to cancer therapy. *FASEB J.* 4, 188–193 (1990).
 68. Wallace, P.M. and Senter, P.D. In vitro and in vivo activities of monoclonal antibody-alkaline phosphatase conjugates in combination with phenol mustard phosphate. *Bioconjug. Chem.* 2, 349–352 (1991).
 69. Vrudhula, V.M., Svensson, H.P., and Senter, P.D. Cephalosporin derivatives of doxorubicin as prodrugs for activation by monoclonal antibody-beta-lactamase conjugates. *J. Med. Chem.* 38, 1380–1385 (1995).
 70. Rodrigues, M.L., Carter, P., Wirth, C., Mullins, S., Lee, A., and Blackburn, B.K. Synthesis and beta-lactamase-mediated activation of a cephalosporin-taxol prodrug. *Chem. Biol.* 2, 223–227 (1995).
 71. Vrudhula, V.M., Svensson, H.P., and Senter, P.D. Immunologically specific activation of a cephalosporin derivative of mitomycin C by monoclonal antibody beta-lactamase conjugates. *J. Med. Chem.* 40, 2788–2792 (1997).
 72. Senter, P.D., Su, P.C., Katsuragi, T., Sakai, T., Cosand, W.L., Hellstrom, I., and Hellstrom, K.E. Generation of 5-fluorouracil from 5-fluorocytosine by monoclonal antibody-cytosine deaminase conjugates. *Bioconjug. Chem.* 2, 447–451 (1991).
 73. Vitols, K.S., Haag-Zeino, B., Baer, T., Montejano, Y.D., Huennekens, F.M. Methotrexate-alpha-phenylalanine: Optimization of methotrexate prodrug for activation by carboxypeptidase A-monoclonal antibody conjugate. *Cancer Res.* 55, 478–481 (1995).
 74. Wolfe, L.A., Mullin, R.J., Laethem, R., Blumenkopf, T.A., Cory, M., Miller, J.F., Keith, B.R., Humphreys, J., and Smith, G.K. Antibody-directed enzyme prodrug therapy with the T268G mutant of human carboxypeptidase A1: In vitro and in vivo studies with prodrugs of methotrexate and the thymidylate synthase inhibitors GW1031 and GW1843. *Bioconjug. Chem.* 10, 38–48 (1999).

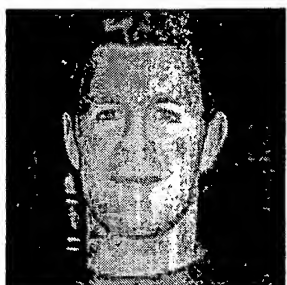
Review



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